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LOMA LINDA UNIVERSITY
School of Behavioral Health
in conjunction with the
Faculty of Graduate Studies

The Effects of Seizure Modeling and Polyphenols on Behavior in
Bang-Sensitive *Drosophila*

by

Alphonso A. Smith

A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Clinical Psychology

September 2017

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Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

<i>bss</i>	<i>bang senseless</i>
cm	centimeters
EA	Ellagic Acid
<i>eas</i>	<i>easily shocked</i>
FL	Flumazenil
PTX	Picrotoxin
s	Seconds
SLA	Seizure-like Activity

ABSTRACT OF THE DISSERTATION

The Effects of Seizure Modeling and Polyphenols on Behavior in Bang-Sensitive *Drosophila*

by

Alphonso A. Smith

Doctor of Philosophy, Graduate Program in Clinical Psychology
Loma Linda University, September 2017
Dr. Richard E. Hartman, Chairperson

Epilepsy is a worldwide public health concern associated with debilitating neurological, cognitive, and psychosocial consequences. Current antiepileptic drugs can have debilitating side effects and new treatments are needed for individuals with pharmaco-resistant seizures like those observed in temporal lobe epilepsy (TLE). Ellagic acid (EA), a polyphenol present in several fruits and nuts, has shown beneficial effects in rodent models of epilepsy and anxiety, possibly mediated through GABAergic pathways. Bang-Sensitive *Drosophila* mutants model epileptic seizures following mechanical shock and provide a high-throughput alternative to costly rodent studies. The objectives of this study were to: 1) examine the effectiveness of seizure modeling in two Bang-Sensitive mutants strains [*bang senseless (bss)* and *easily shocked (eas)*], 2) explore the anticonvulsant effects of EA, and 3) investigate EA's mechanism of action through the addition of two types of GABA antagonists: flumazenil (FL) and picrotoxin (PTX). The results indicated that vortexing consistently induced seizure-like activity (SLA) in both mutant strains. When examining control groups, *bss* flies exhibited more severe SLA when compared to *eas* flies. EA slightly reduced SLA in *bss* flies while both GABA antagonists increased SLA; however these effects were non-significant. *Eas* flies

receiving EA exhibited significantly less SLA when compared to *bss* flies. Both GABA antagonists significantly increased SLA in *eas* flies, with PTX exerting the strongest effect. This possibly indicates that EA's anticonvulsant effect involves GABAergic systems in a strain-specific manner and may be more likely mediated through a GABA_A receptor site than benzodiazepine site. Moreover, utilizing Bang-Sensitive mutants with varying levels of seizure-sensitivity provides a valuable tool for screening plant-based compounds that can be utilized in the treatment of refractory TLE.

CHAPTER ONE

INTRODUCTION

What are Seizures?

According to the International League Against Epilepsy (ILAE) and International Bureau of Epilepsy (IBE), an epileptic seizure is “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher et al., 2005).” Seizures can involve one (focal) or both hemispheres (generalized) of the brain, be accompanied with (complex) or without (simple) an altered state of consciousness, and be convulsive or non-convulsive (Kammerman & Wasserman, 2001). Seizures can also be induced by various triggers such as visual, somatosensory, and complex stimuli as well as by higher-ordered cerebral functions (Striano, Coppola, del Gaudio, & Striano, 2012). The six types of generalized seizures are absence, myoclonic, clonic, tonic, tonic-clonic, and atonic seizures (Kammerman & Wasserman, 2001).

What is Epilepsy?

The ILAE defines epilepsy as a neurological disease characterized by “at least two unprovoked (or reflex) seizures occurring less than 24 hours apart, one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the next 10 years, or a diagnosis of an epilepsy syndrome (Fisher et al., 2014).” In addition, status epilepticus, which can be life threatening, occurs when there is 5 or more minutes of constant seizure activity or recurrent seizure activity without recovery to baseline between seizures

(Brophy et al., 2012).

This disease is a worldwide public health concern that affects approximately 65 million people (Thurman et al., 2011), as it is the third most common neurological disease after Alzheimer's disease and stroke (Kammerman & Wasserman, 2001). Moreover, the incidence of epilepsy and median prevalence rates for lifetime epilepsy and active epilepsy are higher in developing countries in comparison to developed countries (Ngugi, Bottomley, Kleinschmidt, Sander, & Newton, 2010; Thurman et al., 2011). In terms of medically refractory seizures, 30-40% of cases are in developed countries and individuals with epilepsy in developing countries may be at-risk for not receiving any medical treatment (Szilagyi, Szava, Metz, Mihaly, & Orban-Kis, 2014). As such, the need for effective treatments is an imperative concern for industrialized and non-industrialized societies.

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults (Morgan, Conrad, Abou-Khalil, Rogers, & Kang, 2015) as well the most drug-resistant (Freiman, Eismann-Schweimler, & Frotscher, 2011). Furthermore, TLE is associated with damage to multiple brain structures (Bonilha et al., 2010; Focke et al., 2008), cognitive deficits (Alessio et al., 2006; Kaaden & Helmstaedter, 2009), psychiatric disorders (Garcia, 2012; Sanchez-Gistau et al., 2010), and impaired social functioning (Gois et al., 2011; Realmuto et al., 2015). Uncontrolled seizures in TLE also places individuals at an increased risk for death (Choi et al., 2008). The multifaceted consequences of TLE highlight the need for effective treatments for these pharmaco-resistant seizures.

Current Treatments for TLE

Antiepileptic Drug Targets

According to Ojemann (1997), the first line of symptom management with TLE patients is with antiepileptic drugs (AEDs). As such, the commonly used targets/mechanisms for AEDs are reviewed below.

Voltage-Gated Sodium Channels

Voltage-gated sodium channels play a significant role in the generation and conduction of action potentials and ultimately the cellular hyper-excitability and release of neurotransmitters observed in TLE (Hargus et al., 2011; Potschka, 2013). More specifically, when the neuronal membrane is at rest most of the voltage-gated sodium channels are closed. Once a change in membrane potential reaches the threshold potential depolarization occurs and the voltage-gated sodium channel opens allowing sodium ions to rapidly enter the intracellular space (Kwan, Sills, & Brodie, 2001; Potschka, 2013). Next, the voltage-gated channel closes and is not readily re-activated (Kwan et al., 2001). As the membrane is repolarized the voltage-gated sodium channel reverts back to resting potential allowing for future de-polarizations (Kwan et al., 2001).

These voltage-gated sodium channels can cycle through these active and inactive states in milliseconds (fast-activation) or in seconds to minutes (slow-activation) as this plays an important role in the production of epileptic activity (Kwan et al., 2001; Potschka, 2013). Consequently, various AEDs have been developed to target these types of ion channels (Potschka, 2013). Drugs such as carbamazepine, oxacarbazepine, eslicabazepine acetate, phenytoin, lamotrigine, zonisamide, and felbamate promote fast

inactivation by blocking voltage-gated sodium channels. Conversely, lacosamide promotes slow inactivation (Potschka, 2013). As a result, the promotion of these effects is thought to protect against partial and generalized tonic-clonic seizures (Rogawski & Loscher, 2004).

Voltage-Gated Calcium Channels

Similarly to sodium channels, voltage-gated calcium channels are also opened by depolarization of the neuronal membrane and allow rapid influx of calcium ions (Rogawski & Loscher, 2004). These voltage-gated calcium channels can be classified as low voltage and high voltage (Potschka, 2013). The T-type (low voltage-gated calcium channels) contribute to epileptic discharges observed in sub-regions of the thalamus during absence seizures (Potschka, 2013). AEDs such as ethosuximide, valproate, and zonisamide are thought to exert their anti-epileptic properties through blocking these channels (Potschka, 2013).

On the other hand, numerous high voltage-gated calcium channels (L, R, P/Q, N) are located on presynaptic terminals of neuronal axons and have been associated with the release of neurotransmitters that contribute to epileptic activity (Potschka, 2013). AEDs such as felbamate, gabapentin, lamotrigine, levetiracetam, phenobarbital, pregabalin, and topiramate are thought to exert antiepileptic effects by blocking these high voltage-gated calcium channels (Potschka, 2013).

GABAergic Systems

GABA is widely recognized as the most important inhibitory neurotransmitter in

the brain as it plays a vital role inhibiting epileptic activity in areas that vulnerable to epileptic discharges such as the hippocampus, amygdala, and neocortex (Potschka, 2013; Rogawski & Loscher, 2004). GABA exerts its inhibitory effects by binding to fast acting, ionotropic GABA_A receptors (ligand-gated ion channels), which allows for influx of chloride ions resulting in neuronal hyperpolarization and reduction in excitability (Kwan et al., 2001; Potschka, 2013; Rogawski & Loscher, 2004). AEDs such benzodiazepines, felbamate, phenobarbital, retigabine, and topiramate are thought to bind to these receptors (Potschka, 2013).

GABA can also exert its inhibitory effect by binding to slower acting, GABA_B metabotropic G-protein coupled receptors, which can lead to inhibition of voltage-gated calcium channels and/or the increased conductance of potassium ions thereby reducing excitability (Kwan et al., 2001; Potschka, 2013). Other AEDs exert their inhibitory effect by targeting enzymes or transporters that affect the reuptake of GABA in presynaptic neurons or nearby glia cells (Potschka, 2013; Rogawski & Loscher, 2004). For example, vigabatrin inhibits GABA transaminase from degrading GABA in presynaptic neurons or neighboring glia cells which increases the concentration of GABA in the brain (Potschka, 2013). Tiagabine potentiates post-synaptic GABAergic potentials by inhibiting reuptake in presynaptic neurons and nearby glia cells through targeting the GABA transporter, GAT-1 (Potschka, 2013). Lastly, AEDs like gabapentin and valproate increase GABA concentration in the brain by promoting GABA turnover, while levetiracetam affects of modulation of GABA_A receptors (Potschka, 2013).

Glutamatergic Systems

In contrast to GABA, glutamate is widely recognized as the most important excitatory neurotransmitter in the brain (Kwan et al., 2001). Glutamate promotes fast excitatory neurotransmission by binding to ionotropic glutamate receptors such as AMPA, kainate, and NMDA receptors (Potschka, 2013). Moreover, AMPA and kainate receptors are permeable for sodium and potassium, while NMDA receptors are permeable to sodium, potassium, and calcium (Potschka, 2013). Consequently, the binding of glutamate to these receptors results in the influx of cations into post-synaptic neurons which leads to neuronal excitability (Potschka, 2013). This is consistent with research demonstrating that focal injections of glutamate result in seizures (Kwan et al., 2001). Clinical efficacy for a selective glutamate receptor antagonist has not been demonstrated as of yet. However, AEDs with multiple mechanisms of actions such as felbamate, topiramate, and phenobarbital may have a glutamatergic effect (Potschka, 2013).

Resective Surgery and Additional Treatments

As TLE is the form epilepsy that is the most drug-resistant to seizures, other techniques such as resective surgery of the temporal lobe has been utilized to treat patients (Engel et al., 2012). Wiebe, Blume, Girvin, and Eliasziw (2001) in a randomized controlled trial demonstrated that resective surgery was superior to prolonged AED therapy in terms of reduced seizures and improved quality of life among TLE patients with poorly controlled seizures. Expanding on this study, Engel et al. (2012) also performed a randomized clinical trial with TLE patients that had drug-resistant seizures. The results showed that patients who underwent resective surgery with continued AED

treatment experienced significantly less seizures than individuals who only received AED treatment (Engel et al., 2012). In addition to surgery, other treatments options including vagus nerve stimulation (Aihua et al., 2014; Marras et al., 2013; Waseem, Raffa, Benbadis, & Vale, 2014) and the ketogenic diet (Pfeifer & Thiele, 2005; Sirven et al., 1999) have demonstrated some benefits for the treatment of epilepsy.

Rodent Models of TLE and Seizures

An array of animal models (e.g. cats, dogs, non-human primates, fish, worms, and flies) have been used to study seizures and/or epilepsy (Grone & Baraban, 2015). However, rodent models are the more commonly used and widely recognized paradigm for modeling seizures and TLE (Grone & Baraban, 2015). As a result, the major techniques will be examined further.

Chemoconvulsant Models

Chemoconvulsants such as kainic acid and pilocarpine are used in rodents to model TLE through an initial injury such as status epilepticus, which is followed by a latent period before the onset spontaneous chronic seizures (Kandratavicius et al., 2014). The seizures are typically partial and tonic-clonic type while histopathological changes are reflective of TLE (Kandratavicius et al., 2014). Kainic acid is a L-glutamate analog and agonist for ionotropic kainate receptors in the hippocampus (Kandratavicius et al., 2014; Levesque & Avoli, 2013). Injected rodents also typically develop degenerated brain tissue that models TLE hippocampal sclerosis (Kandratavicius et al., 2014).

Injecting pilocarpine, a muscarinic acetylcholine receptor agonist, results in

hippocampal damage and produces lesions in the neocortex (Buckmaster, 2004; Kandratavicius et al., 2014). Lastly, both methods can be administered systemically or intrahippocampally (Kandratavicius et al., 2014; Levesque & Avoli, 2013). However, the drawbacks for both of these chronic epilepsy models are the experimental costs and time constraints (Kandratavicius et al., 2014). Other convulsant agents such as pentylenetetrazol (PTZ), strychnine, N-methyl-D, or L-aspartate can be used to screen AEDs in acute seizure models as opposed to chronic epilepsy (Kandratavicius et al., 2014).

Electrical Stimulation Models

In addition to chemoconvulsants, models such as electroshock-induced seizures and kindling have been used to study seizures (Kandratavicius et al., 2014). In the electroshock model, mild stimulation from corneal electrodes result in myoclonic seizures, while more intense stimulation can lead to generalized tonic-clonic seizures (Kandratavicius et al., 2014). This approach has been used to research how epileptic discharges can adversely impact synaptic plasticity, cognition, and co-morbid psychiatric conditions (Kandratavicius et al., 2014).

The kindling method involves continuous, mild electrical stimulation of regions such the hippocampus, amygdala, and olfactory areas and other brain regions (Buckmaster, 2004). This can result in partial seizures leading to secondary generalization and spontaneous seizures (Kandratavicius et al., 2014). The advantages to these two models are the low rates of mortality and high reproducibility of findings. The disadvantages arise when these models are applied for chronic seizure research as the

process can become expensive and labor-intensive (Kandratavicius et al., 2014).

Brain Pathology Models

Utilizing rodents also allows for modeling of febrile seizures, neonatal hypoxic encephalopathy, and post-traumatic epilepsy. For example, raising the body temperature of immature rodents results in hyperthermic seizures that model febrile seizures observed in childhood (Kandratavicius et al., 2014). The behavioral manifestation of these seizures are characterized by immobility, facial automatisms, and myoclonic jerking (Kandratavicius et al., 2014). The hypoxia model exposes immature rodents to air concentrations with low amounts of oxygen, which results in brief and repetitive tonic-clonic seizures mimicking neonatal hypoxic encephalopathy (Kandratavicius et al., 2014). The post-traumatic epilepsy model induces brain damage through a fluid percussion injury that later results in low frequency generalized tonic-clonic seizures (Kandratavicius et al., 2014).

Genetic Models

Genetic models have also been used to study various types of epilepsy. More specifically, audiogenic seizures have been used to model not only reflex epilepsy, but also TLE as well (Kandratavicius et al., 2014). These seizures are induced by high-intensity acoustic stimuli that result in wild running and tonic-clonic seizures (Kandratavicius et al., 2014). Various rodent strains such as Genetically Epilepsy-Prone Rats (GEPRs), P77PMC rats, Wistar Albino Glaxo/Rijwijk rats (WAG/Rij), Wistar Audiogenic Sensitive Rats, and Wistar Audiogenic Rats (WAR) have been used in

audiogenic seizure experiments (Kandratavicius et al., 2014). Limitations of this model include requiring specific auditory stimuli to evoke seizure activity and the lack of recurrent chronic seizures (Kandratavicius et al., 2014).

Drosophila Melanogaster as a Research Model

Although rodent models have been effective in modeling seizures and epilepsy, the experiments and maintenance can be cost-prohibitive and very lengthy. On the hand, seizure modeling in *Drosophila melanogaster* is appealing due to the small size, low cost, and rapid transgenesis of this organism (Grone & Baraban, 2015). Additionally, *Drosophila* have 4 chromosomes and about 14,000 genes with limited redundancy in comparison to rodents, which makes it easier to study a particular gene's function ("JoVE," 2015). More advantages include a short life cycle (approximately 2 weeks) that has four major stages (embryo, larva, pupa, adult) and a 60-80 day lifespan depending on environmental conditions ("JoVE," 2015). In terms of offspring, a fertile mating pair can produce a genetically identical progeny of hundreds within the span of 10-12 days at 25 °C, while rodent models can only produce a limited amount of offspring every 3 to 4 months (Pandey & Nichols, 2011).

With respect to the adult fruit fly brain, *Drosophila* have about 100,000 neurons that have distinct connections that mediate a variety of behaviors such as learning and memory, courtship, aggression, feeding, grooming, circadian rhythms, sleep, and flight navigation (Pandey & Nichols, 2011). Notably, voltage-gated and ligand-gated channels have highly conserved homologues in *Drosophila* (Parker, Howlett, Rusan, & Tanouye, 2011). GABA, glutamate, and acetylcholine neurotransmitter receptors also

have highly conserved homologues (Parker, Howlett, et al., 2011). Moreover, about 50% of this species' genes originate from a common ancestor as humans and 75% of human-disease related genes are orthologous or function similarly in *Drosophila* ("JoVE," 2015). As such, these advantages make *Drosophila* a viable alternative to rodents for studying pharmaco-resistant epilepsy and testing anticonvulsants.

Seizure Modeling in *Drosophila Melanogaster*

The Bang-Sensitive class of *Drosophila* mutants experience seizure-like activity (SLA) following mechanical stimuli that can be used to model intractable epilepsy in humans. Following mechanical shock either by tapping or vortexing a vial, Bang-Sensitive mutants undergo an initial seizure (2 seconds), temporary paralysis (20-300 seconds), and then a recovery seizure (2 seconds) (Song & Tanouye, 2008) (see Figure 1). Lastly, there is a refractory period where seizures cannot be induced and then a complete recovery period where the mutants regain seizure-sensitivity (Parker, Howlett, et al., 2011) (see Figure 1). SLA in Bang-Sensitive mutants can also be induced through high frequency electrical stimulation, as they have much lower electrophysiological seizure thresholds (usually 10 V) than their wild-type counterparts (usually 30 V) (Song & Tanouye, 2008).

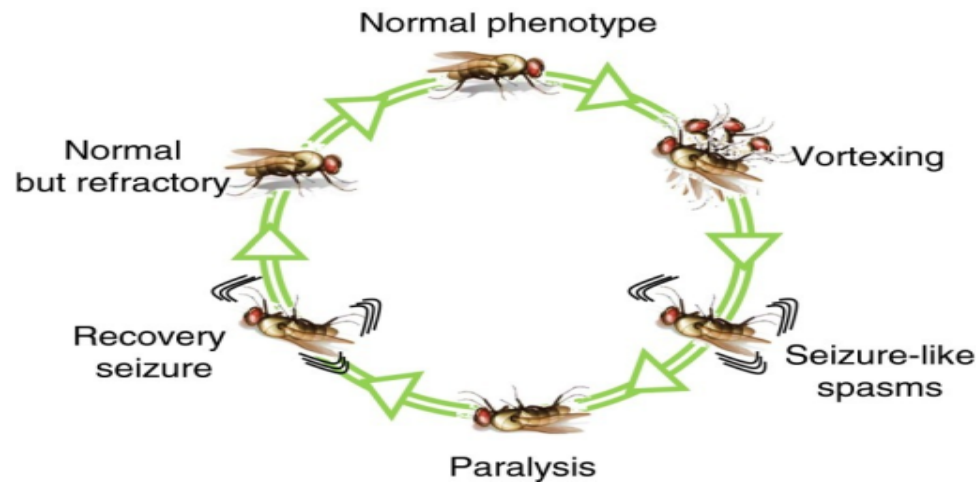


Figure 1. Following mechanical shock Bang-Sensitive mutants undergo an initial seizure, temporary paralysis, and then a recovery seizure before returning to baseline. Image from “Regulation of membrane excitability: A convergence on voltage-gated sodium conductance,” by W. Lin and R. A. Baines, 2015, *Molecular Neurobiology*, 51, p. 60. © The Author(s) 2014

There are six canonical mutant strains which include *bang senseless* (*bss*), *bang sensitive* (*bas*), *easily shocked* (*eas*), *slamdance* (*sda*), *technical knockout* (*tko*), and *knockdown* (*kdn*) (Song & Tanouye, 2008). The *bss*¹ and *eas*² strains are completely penetrant with respect to expressing seizure-like activity (Song & Tanouye, 2008) and were utilized in this study. The *bss*¹ strain has a mutation that affects voltage-gated sodium channels by shifting the voltage of fast inactivation to more positive potentials without any effect on activation voltage (Parker, Padilla, Du, Dong, & Tanouye, 2011). This results in tonic-clonic seizure-like activity following mechanical shock, as this mutant is the most severe of the Bang-Sensitive mutants (Song & Tanouye, 2008). Moreover, the *bss* strain is viewed as a model of intractable epilepsy due to the difficulty to pharmacologically and genetically suppress the strain’s severe seizure-sensitivity

(Parker, Padilla, et al., 2011; Song & Tanouye, 2008).

Mutants from the *eas*² strain have an ethanolamine kinase defect that impairs the synthesis of phosphatidylethanolamine, which is a primary membrane lipid in *Drosophila* (Pavlidis, Ramaswami, & Tanouye, 1994; Song & Tanouye, 2008). As a result, this defect is thought to increase hyper-excitability by affecting integral membrane proteins such as ion channels or through membrane fusion events like neurotransmitter secretion as these mutants display moderate seizure sensitivity (Pavlidis et al., 1994; Song & Tanouye, 2008).

Anticonvulsant Studies with *bss* and *eas* Mutants

As SLA in Bang-Sensitive mutants models drug-resistant epilepsy in humans, multiple experiments with *bss* and *eas* mutants have been performed to screen AED treatments (Baraban, 2007). Kuebler and Tanouye (2002) demonstrated that head injections of valproate reduced seizure-sensitivity in *bss* mutants during high frequency electrical stimulation. Next, Reynolds et al. (2004) examined seizure modeling after treating *eas* and *bss* strains with a range of AEDs. For *eas* mutants, Reynolds et al. (2004) observed that acute feeding with AEDs such as carbamazepine, ethosuximide, and vigabatrin had no significant effect on seizure recovery time following mechanical shock. However, acute feeding with phenytoin at a range doses significantly reduced seizure recovery time for *eas* mutants (Reynolds et al., 2004). For chronic feeding, phenytoin and gabapentin at a range of doses significantly reduced seizure recovery time following mechanical shock, while ethosuximide only had a significant effect at a higher dosage (Reynolds et al., 2004). Additionally, chronic exposure to phenytoin reduced seizure

sensitivity to high frequency electrical stimulation (Reynolds et al., 2004). For *bss* mutants, feeding gabapentin reduced seizure recovery time in a strain-specific manner, while phenytoin's anticonvulsant effect was dose-dependent (Reynolds et al., 2004).

In another study, Tan, Lin, and Tanouye (2004) examined the anticonvulsant effects of potassium bromide on *bss* and *eas* strains. The results demonstrated that there was significant improvement for seizure recovery time in response to mechanical and electrical stimulation for *bss* mutants as these effects were dependent on the concentration and feeding duration of potassium bromide; however, this AED was ineffective for *eas* mutants (Tan et al., 2004).

Stone, Evans, Coleman, and Kuebler (2013) employed genetic and non-traditional pharmacological manipulations to up-regulate metabolism in *bss* and *eas* mutants to reduce SLA. More specifically, they introduced the *atusugari* (*atu*) mutation to increase the metabolic rate (Stone et al., 2013). After receiving mechanical shock, both strains with the *atu* mutation exhibited reduced SLA (Stone et al., 2013). *Eas* mutants with the *atu* mutation also displayed increased levels of metabolism in comparison to their single mutant counterparts (Stone et al., 2013).

With respect to the drug treatment, both strains were fed tolbutamide, which is associated with increased hemolymph and lipid metabolism (Stone et al., 2013). The results demonstrated that *eas* flies exhibited reduced SLA and faster recovery following a mechanical shock in comparison to their counterparts that did not receive the drug (Stone et al., 2013). On the other hand, the *bss* flies did not experience a significant reduction in SLA, but did exhibit faster recovery times in comparison to *bss* flies that did not receive tolbutamide (Stone et al., 2013). Lastly, Stone, Burke, Pathakamuri, Coleman, and

Kuebler (2014) demonstrated that the *eas* mutants who were fed metformin (used to treat type II diabetes) exhibited reduced SLA.

In summary, these studies demonstrate that the standard AEDs and non-traditional treatments used to treat intractable epilepsy in humans have varying levels of efficacy in Bang-Sensitive mutants. Consequently, developing new and innovative types of anticonvulsive treatments in Bang-Sensitive mutants will have important research implications for drug-resistant seizures observed in TLE patients. More specifically, new treatments could possibly mitigate the neurological, cognitive, and psychological outcomes in TLE and reduce increased risk for death associated with uncontrolled seizures. Additionally, individuals treated with the commonly used AEDs can have neurocognitive side effects (Arif et al., 2009; Javed et al., 2015; Kwan & Brodie, 2001; Ortinski & Meador, 2004) as well as psychiatric and behavioral problems (Weintraub, Buchsbaum, Resor, & Hirsch, 2007), which further highlights the need for additional treatments. As a result, a novel and alternative anticonvulsive treatment for the two Bang-Sensitive mutants in this prospective study will be discussed next.

Properties of Ellagic Acid

Ellagic acid (EA), a naturally occurring polyphenol, is present in a variety of fruits and nuts including pomegranates (Panichayupakaranant, Itsuriya, & Sirikatitham, 2010), Indian gooseberries (Poltanov et al., 2009), raspberries (Bobinaitė, Viškelis, & Venskutonis, 2012), strawberries (Kosmala et al., 2014), walnuts (Anderson et al., 2001), pecans (Malik et al., 2009), and chestnuts (Gonçalves et al., 2010). Furthermore, EA appears to have neuro-protective (Dolatshahi, Farbood, Sarkaki, Mansouri, & Khodadadi,

2015; Farbood et al., 2015), anti-inflammatory (Farbood et al., 2015; Papoutsi et al., 2008), antioxidant (Al-Obaidi et al., 2014; Yu, Chang, Wu, & Chiang, 2005), anxiolytic (Girish, Raj, Arya, & Balakrishnan, 2013), and anti-depressant (Dhingra & Chhillar, 2012; Ferreres, Grosso, Gil-Izquierdo, Valentão, & Andrade, 2013) properties.

Potential Mechanism of Action for Ellagic Acid

Research has demonstrated that EA has anticonvulsant properties as well. Dhingra and Jangra (2014) examined the acute and chronic effects of EA in mice following pentylenetetrazol (PTZ) and picrotoxin-induced (PTX) convulsions. PTZ triggers convulsions by exerting an inhibitory effect on GABA-mediated chloride ion influx via an allosteric interaction at chloride ion channels, which leads to the activation of excitatory neurons, excitotoxicity and ultimately seizures (Dhingra & Jangra, 2014). PTX is a non-competitive GABA_A receptor antagonist that blocks chloride ion channels which leads to convulsions as well (Dhingra & Jangra, 2014).

The study demonstrated that acute and chronic administration of EA delayed the onset of convulsions, while also reducing the duration of tonic and clonic convulsions and mortality in both PTZ and PTX conditions (Dhingra & Jangra, 2014). EA significantly increased levels of GABA in the brain as well (Dhingra & Jangra, 2014). As a result, Dhingra and Jangra (2014) asserted that EA's anticonvulsant effects most likely involve GABAergic neurotransmission. Additionally, Girish et al. (2013) demonstrated that EA had an anxiolytic effect on mice that was antagonized by PTX and flumazenil (FL) (a benzodiazepine site antagonist). This further provided evidence that EA possibly exert its effects through the GABAergic system.

Further investigating this potential GABAergic mechanism for EA is critical since decreased levels of GABA in the brain have been linked to poorer seizure control (Petroff, Rothman, Behar, & Mattson, 1996) as hyper-excitability is a defining feature of epilepsy (Fisher et al., 2005). Moreover, utilizing Bang-Sensitive *Drosophila* provide an excellent opportunity to study this mechanism of action since they have highly conserved homologues with humans with respect to GABAergic neurons (Liu, Krause, & Davis, 2007; Parker, Howlett, et al., 2011).

The purpose study of this study was to evaluate the usefulness of seizure modeling in the *bss* and *eas* strains and examine the potential anticonvulsant properties of EA while attempting to block this effect with GABA antagonists (FL and PTX) to better understand its role in GABAergic neurotransmission. This will expand on the prior research performed with Bang-Sensitive mutants as none of the previously cited studies utilized polyphenols or attempted to block the effect of anticonvulsants to clarify the mechanism of action.

Aims of Study

Aim 1: Determine the effects of seizure modeling with mechanical shock in Bang-Sensitive mutants.

Hypothesis: *Vortexing individual bss and eas mutants will induce SLA.* Rationale: Bang-Sensitive mutants display SLA following mechanical shock, which will be assessed in terms of duration, distance moved, and velocity.

Aim 2: Determine the anticonvulsive effects of EA on SLA.

Hypothesis: *Dietary supplementation with EA will reduce the SLA of Bang-Sensitive*

mutants. Rationale: The anticonvulsant properties of EA will reduce SLA with respect to duration, distance moved, and velocity. Two-day old, adult male *bss* and *eas* flies will be fed either standard media or standard media mixed with EA.

Aim 3: Determine if the anticonvulsive effects of EA on SLA can be pharmacologically blocked.

Hypothesis: *The anticonvulsant effects of EA on Bang-Sensitive mutants will be blocked*

by GABA antagonists. Rationale: EA is thought to exert its anticonvulsive properties through GABAergic transmission. As a result, this effect should be blocked by either FL (a benzodiazepine site antagonist) or PTX (non-competitive GABA_A receptor antagonist).

This should result in increased SLA with respect to duration, distance moved, and velocity. Two-day old, adult male *bss* and *eas* flies will be fed standard media mixed with EA + FL or standard media mixed EA + PTX.

CHAPTER TWO

RESEARCH DESIGN

Methods

Fly Stocks

The *bss*¹ and *eas*² strains were obtained from Dr. Daniel Kuebler at the Franciscan University of Steubenville. The stocks were maintained and housed in two population boxes (see Figure 2A). They were cultured on Formula 4-24® Instant *Drosophila* Medium, Blue (Carolina Biological Supply, Burlington, NC) at an average temperature of 75 °F on a 12hr/12hr light-dark cycle (see Figure 2A). Eggs for the experiment were then collected from food plates as needed and placed into vials with 1 teaspoon of media (see Figure 2B). After approximately two weeks, newly eclosed flies were collected and used for the study.

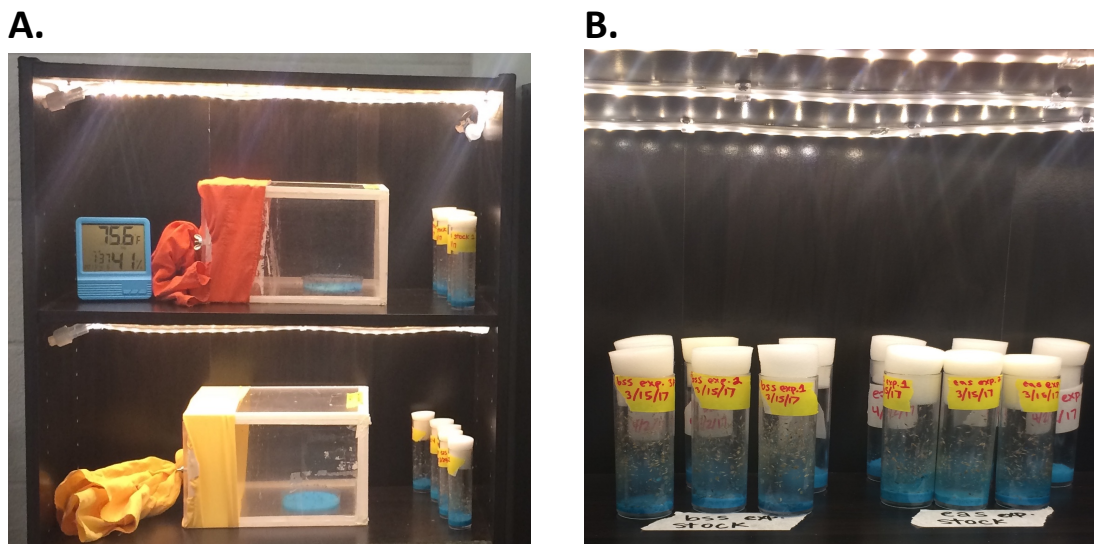


Figure 2. Equipment used for *Drosophila* husbandry. (A) Population boxes along with supplementary vials that were used to maintain the respective mutant stocks. (B) Vials that were used to develop experimental *bss* and *eas* flies for the study.

Selection of Treatment Doses

Pomegranate extract powder (90% ellagic acid) (PureBulk, Roseburg, OR), PTX (INDOFINE Chemical Company, Hillsborough, NJ), and FL (Enzo Life Sciences, Farmingdale, NY) were used for the experiment. The PTX solution was made by dissolving 100 mg of PTX in 1 mL of DMSO and then diluting with 200 mL water. The FL solution was made by dissolving 5 mg of FL in 1 mL of DMSO and then diluting with 1L water. The EA dose was based on prior polyphenol research with *Drosophila* (Wang et al., 2015). Likewise, the PTX dose was based on prior PTX research with *Drosophila* (Stilwell, Saraswati, Littleton, & Chouinard, 2006). The FL dose was derived from research with *Daphnia pulex* (Dong, Hu, Ni, Zuo, & Li, 2013) as there were no studies to date using FL for seizure modeling in *Drosophila*.

Experimental Groups

Newly emerged adult males were given the standard media diet for 48 hours. Next, they were anesthetized with FlyNap and individually placed in vials where they were randomly assigned to receive 48 hours of one of four treatments: control diet ($\frac{1}{2}$ teaspoon of standard media), EA treatment (25 mg of pomegranate extract powder mixed with standard media), EA + FL treatment (2.5 mL of FL solution added to EA and standard media) or EA + PTX treatment (2.5 mL of PTX solution added to EA and standard media). Twenty-eight males were assigned to each diet condition across both strains for a total of 224 flies in the study (see Table 1).

Table 1. Experimental group breakdown.

	Standard Media	EA	EA + FL	EA + PTX
<i>bss</i>	28	28	28	28
<i>eas</i>	28	28	28	28

Seizure-like Activity Assay

This assay was based on the method performed by Stone et al. (2014). After the two-day drug-feeding period, flies were transferred to individual vials where they were undisturbed for least 20-30 minutes. None of the flies were exposed to anesthesia over the prior 48-hour period. Next, individual vials were vortexed on the maximum setting (Scilogex MX-S Vortex Mixer) for 10 seconds (see Figure 3A). Then single flies were placed on a white sheet of paper underneath a webcam (Logitech C270) and covered with a Petri dish with air slits (see Figure 3B). Seizure activity was recorded with HandyAvi

time-lapse picture software. The image stacks were processed using ImageJ multitracker software and yielded raw pixel x-y coordinates for each fly. This data was then converted to seizure information such as duration (s), distance moved (cm), and velocity (cm/s) with a custom Excel Visual Basic Program (Fly Analysis Software) created by Stone et al. (2014).

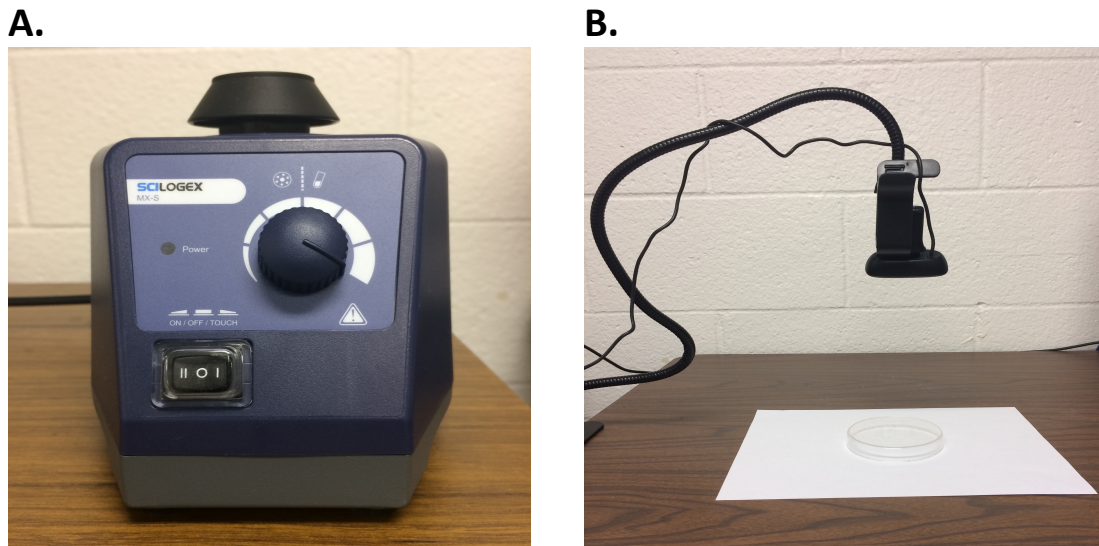


Figure 3. Equipment used for seizure assay. (A) Vortexer on highest setting. (B) Recording setup including webcam, white sheet of paper, and Petri dish.

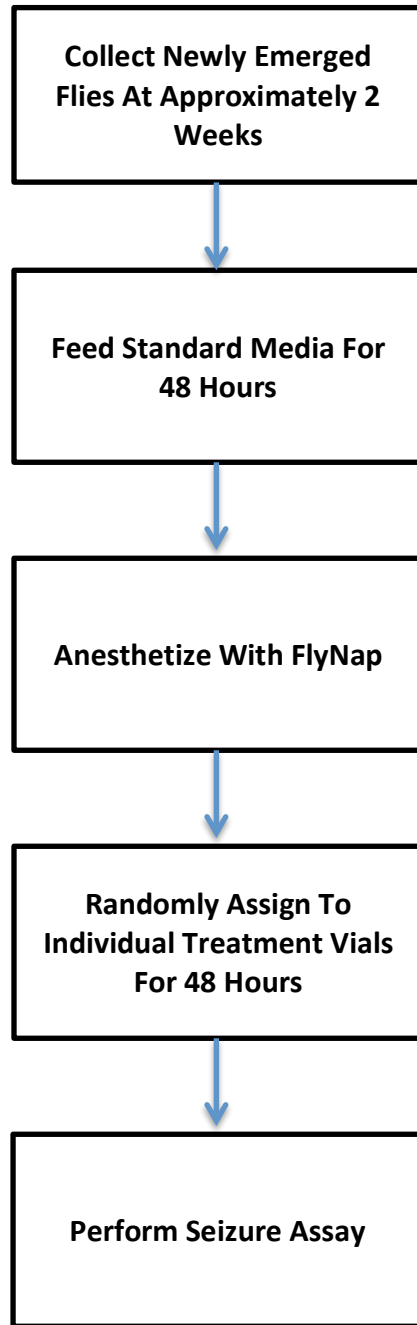


Figure 4. Schematic diagram of experiment.

Statistical Analysis

SPSS was used to perform all of the statistical tests. Prism was used to generate graphs. Three 2-way ANOVAs with Bonferroni post hoc tests were performed with genotype and diet on the dependent variables of duration, distance moved, and velocity. This approach was used instead of a MANOVA due to the linear dependency of velocity with duration of SLA and distance moved during SLA. This method was also utilized over a MANOVA due to the significant, positive correlations between duration of SLA and distance moved during SLA observed in both genotypes and all diet conditions (see Results). Flies that did not exhibit SLA were removed from the analysis ($n = 14$), which resulted in 25-28 flies per diet condition (see Table 2). Chi-Square analyses demonstrated that non-seizing flies that were removed did not differ significantly from the remaining flies with respect to genotype or diet ($p > .05$). The Kolmogorov-Smirnov Test demonstrated that the data met assumptions for normality.

Table 2. Experimental group breakdown with non-seizing flies removed.

	Standard Media	EA	EA + FL	EA + PTX
<i>bss</i>	27 (96.4%)	25 (89.3%)	26 (92.9%)	27 (96.4%)
<i>eas</i>	26 (92.9%)	26 (92.9%)	25 (89.3%)	28 (100%)

Percentage of remaining flies in each condition in parentheses

CHAPTER THREE

RESULTS

Main Effect of Genotype

There was no significant main effect for genotype on duration of SLA as *bss* flies ($M = 6.16$ s) did not differ significantly from *eas* flies ($M = 5.84$ s), $F(1, 202) = .68$, $p > .05$ (see Figure 5).

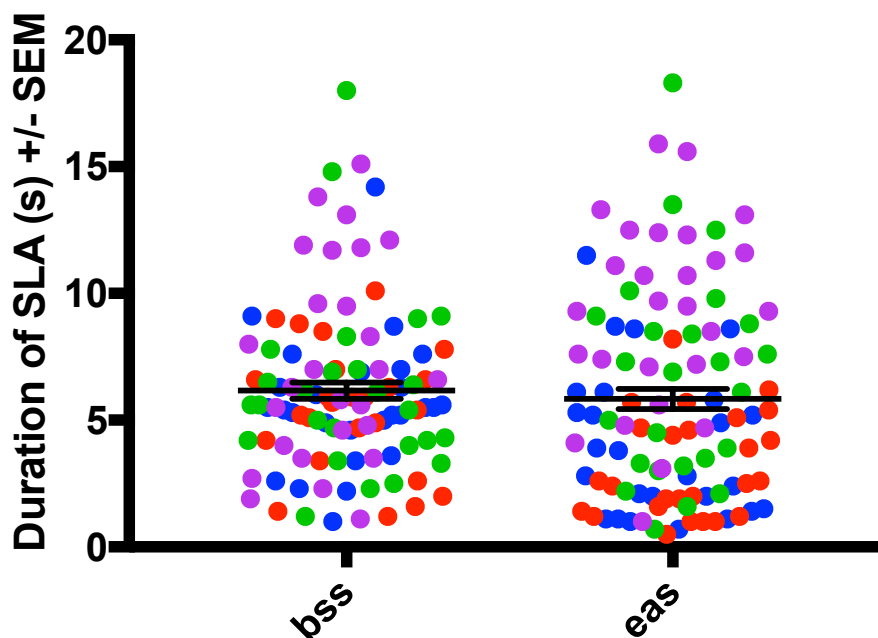


Figure 5. There was no significant difference in duration of SLA between *bss* and *eas* flies. The data point color corresponds to diet condition (blue = control; red = ellagic acid; green = ellagic acid + flumazenil; purple = ellagic acid + picrotoxin).

There was no significant main effect for genotype on distance moved during SLA as *bss* flies ($M = 14.23$ cm) did not differ significantly from *eas* flies ($M = 12.16$ cm), $F(1, 202) = 2.46, p = .12$ (see Figure 6).

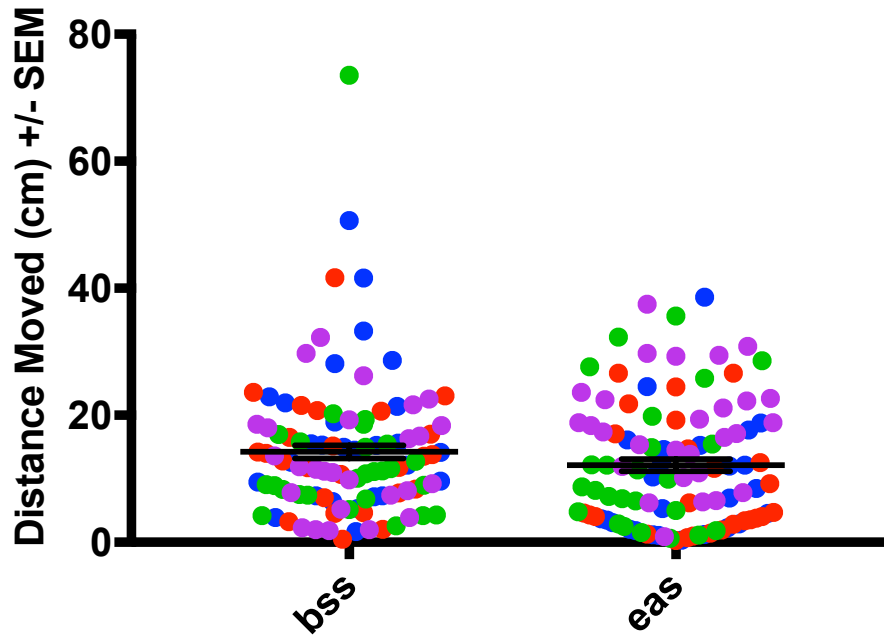


Figure 6. There was no significant difference in distance moved during SLA between *bss* and *eas* flies. The data point color corresponds to diet condition (blue = control; red = ellagic acid; green = ellagic acid + flumazenil; purple = ellagic acid + picrotoxin).

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *bss* flies, $r = .76$, $p < .001$ (see Figure 7).

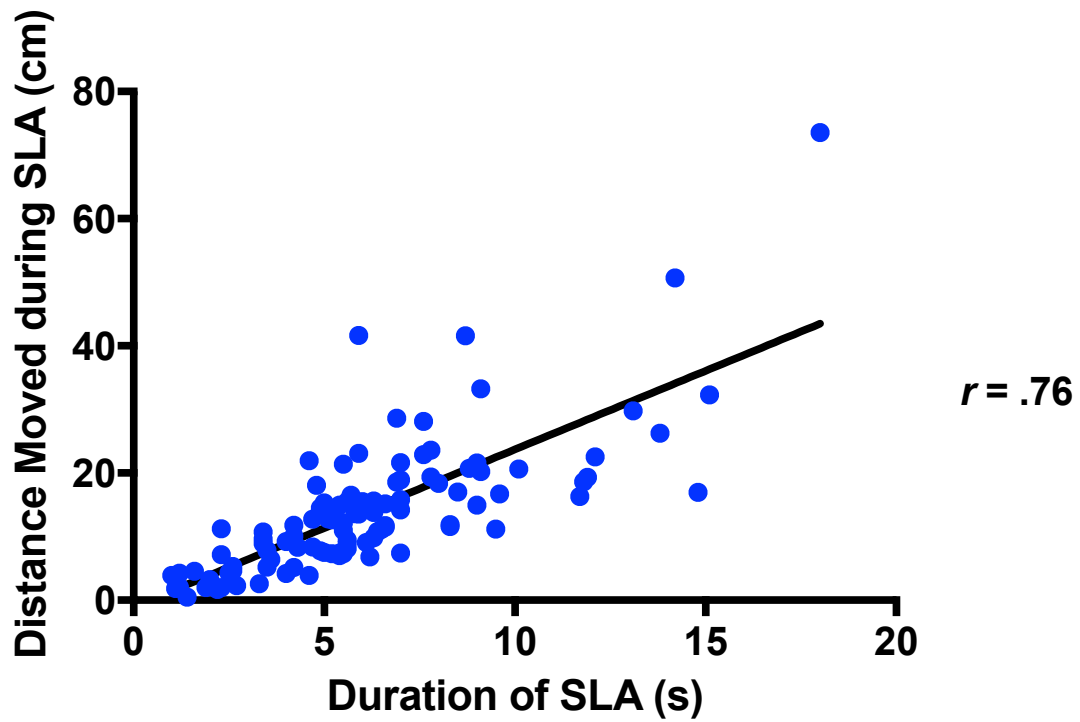


Figure 7. In *bss* flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .76$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *eas* flies, $r = .83$, $p < .001$ (see Figure 8).

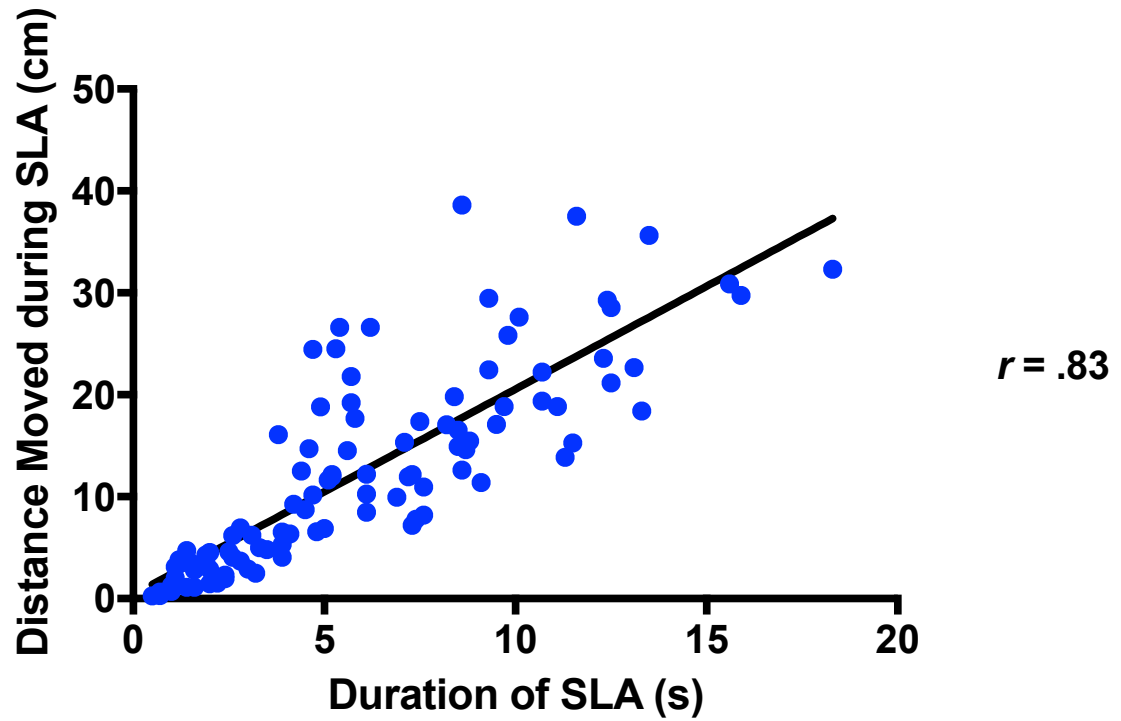


Figure 8. In *eas* flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .83$, $p < .001$.

There was a significant main effect for genotype on velocity of SLA. *Bss* flies ($M = 2.27$ cm/s) had significantly higher velocity of SLA than *eas* flies ($M = 1.98$ cm/s), $F(1, 202) = 5.04, p < .05$ (see Figure 9).

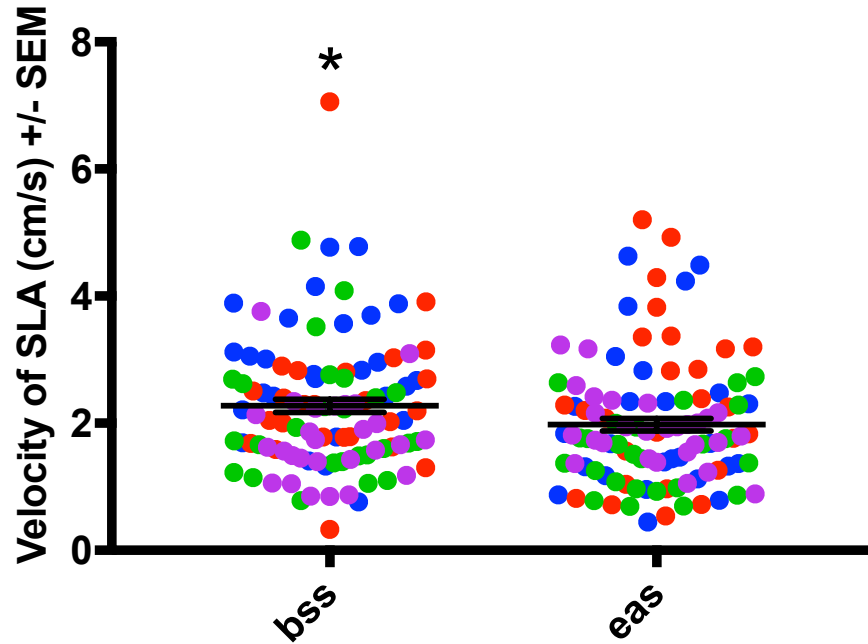


Figure 9. *Bss* flies had significantly higher velocity of SLA than *eas* flies. The data point color corresponds to diet condition (blue = control; red = ellagic acid; green = ellagic acid + flumazenil; purple = ellagic acid + picrotoxin). $*p < .05$

Main Effect of Diet

There was a significant main effect for diet, $F(1, 202) = 15.61, p < .001$. The Bonferroni *post hoc* test revealed that duration of SLA was significantly higher for the EA + PTX condition ($M = 8.25$ s) when compared to the control ($M = 4.87$ s) ($p < .001$), EA ($M = 4.29$ s) ($p < .001$), and EA + FL conditions ($M = 6.45$ s) ($p < .05$) (see Figure 10). Duration of SLA was higher for the EA + FL condition ($M = 6.45$ s) when compared to the EA ($M = 4.29$ s) ($p < .05$) and control ($M = 4.87$ s) ($p < .01$) condition (see Figure 10). Flies in the EA condition ($M = 4.29$ s) did not significantly differ from controls ($M = 4.87$ s) ($p > .05$) (see Figure 10).

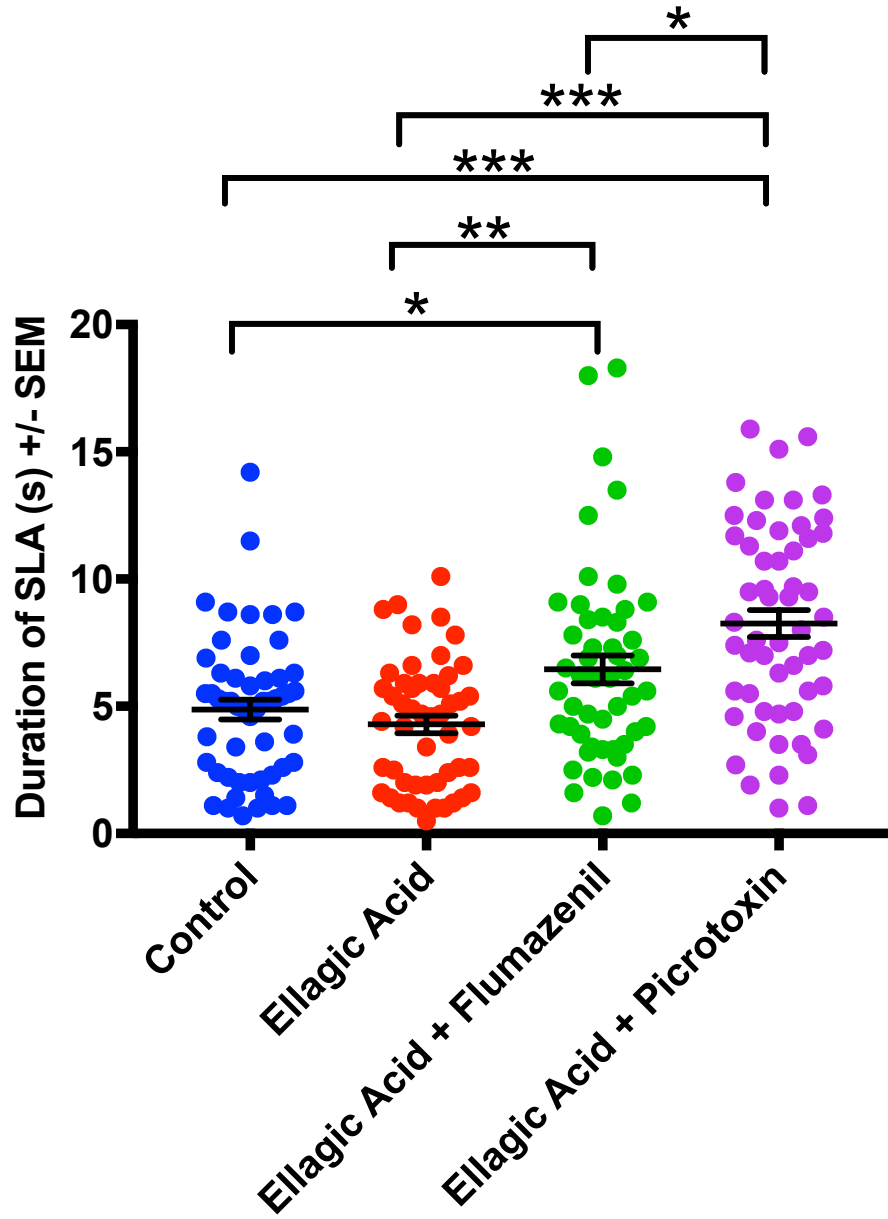


Figure 10. EA + PTX flies had significantly longer duration of SLA when compared to all other conditions. EA + FL flies had significantly longer duration of SLA when compared to the EA and control conditions. Flies on the EA diet did not differ significantly from controls. * $p < .05$; ** $p < .01$; *** $p < .001$

There was no significant main effect for diet. Distance moved during SLA did not differ significantly among the control ($M = 13.14$ cm), EA ($M = 11.22$ cm), EA + FL ($M = 12.62$ cm), and EA + PTX conditions ($M = 15.61$ cm), $F(1, 202) = 2.46$, $p = .12$ (see Figure 11).

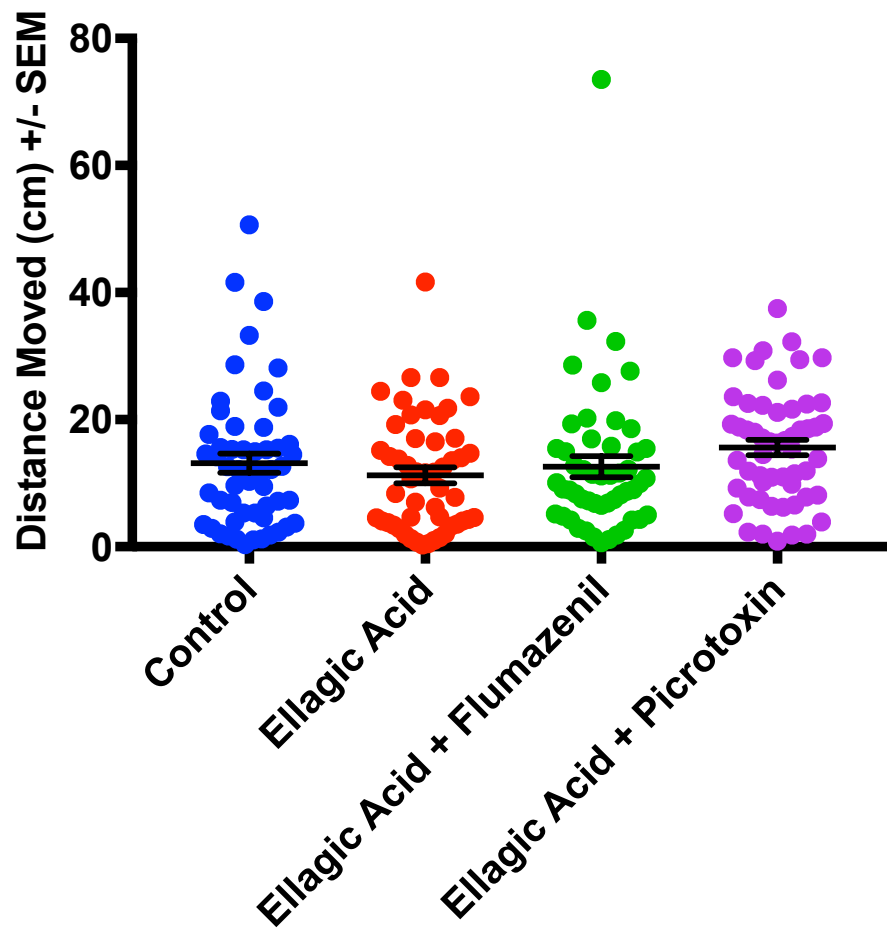


Figure 11. There was no significant difference in distance moved during SLA among diet conditions.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among flies receiving the control diet, $r = .82$, $p < .001$ (see Figure 12).

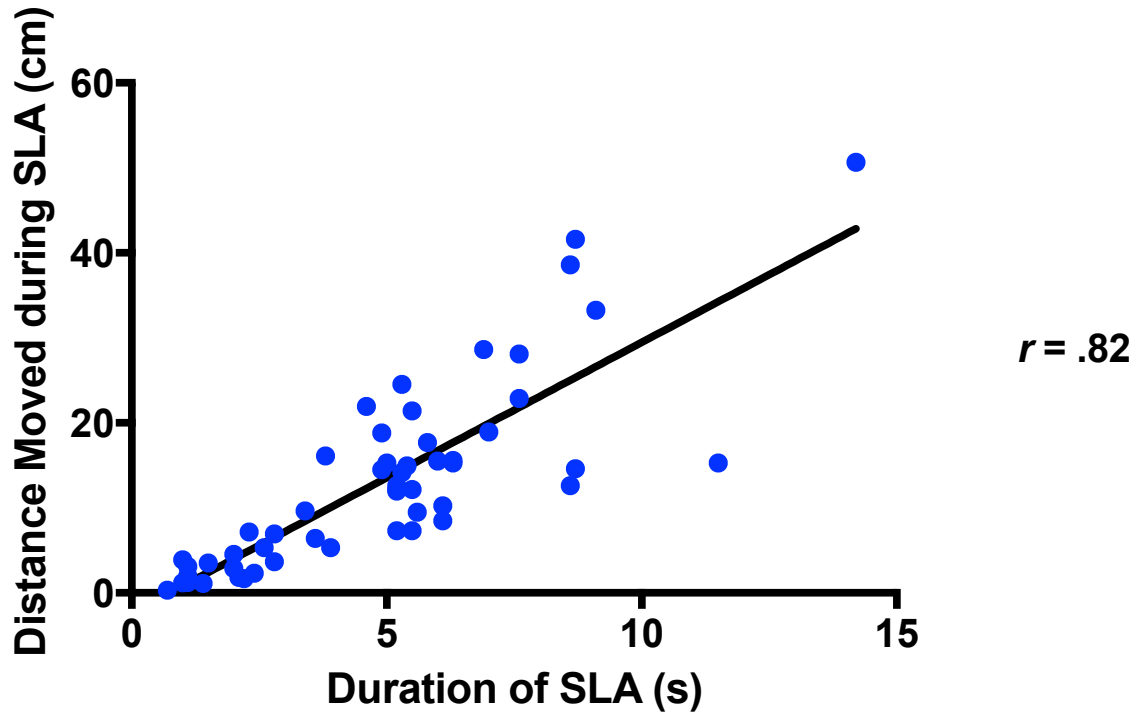


Figure 12. In control flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .82$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among flies receiving EA, $r = .77$, $p < .001$ (see Figure 13).

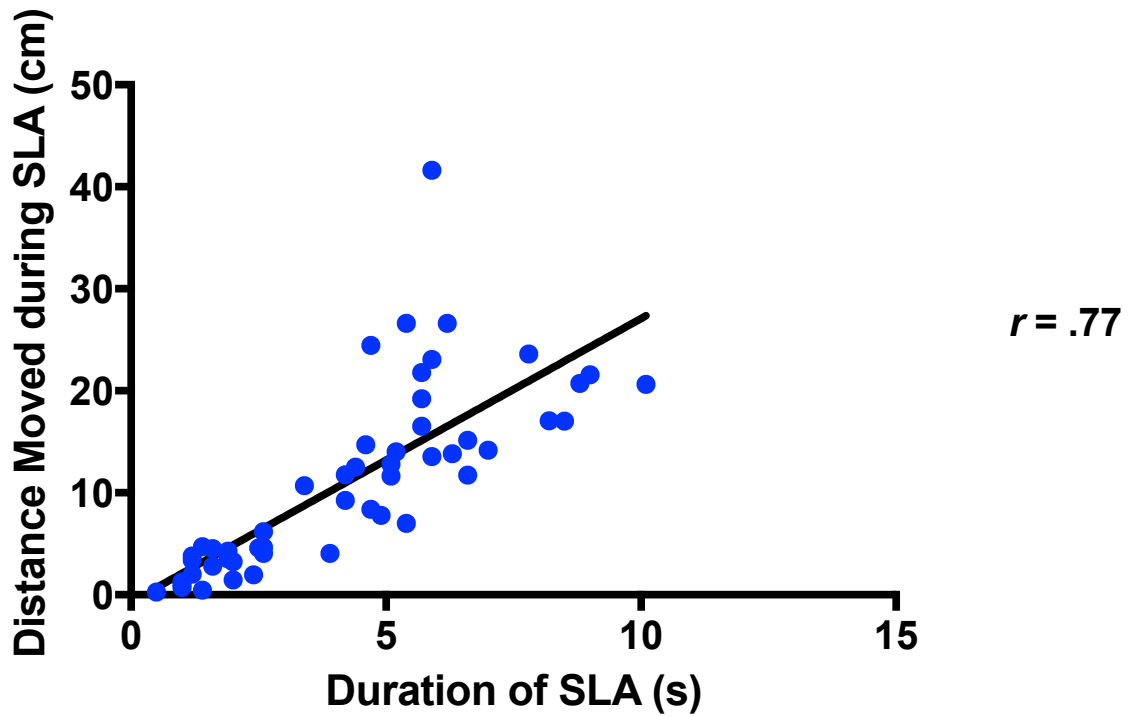


Figure 13. In EA flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .77$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among flies receiving EA + FL, $r = .84$, $p < .001$ (see Figure 14).

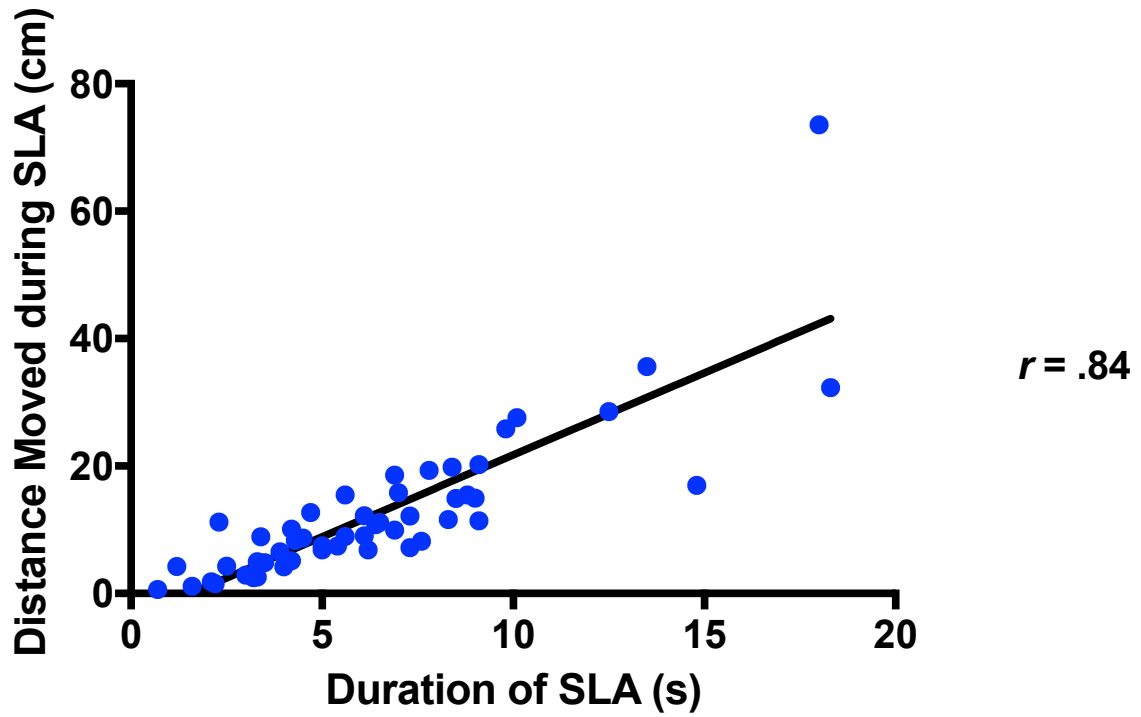


Figure 14. In EA + FL flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .84$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among flies receiving EA + PTX, $r = .86$, $p < .001$ (see Figure 15).

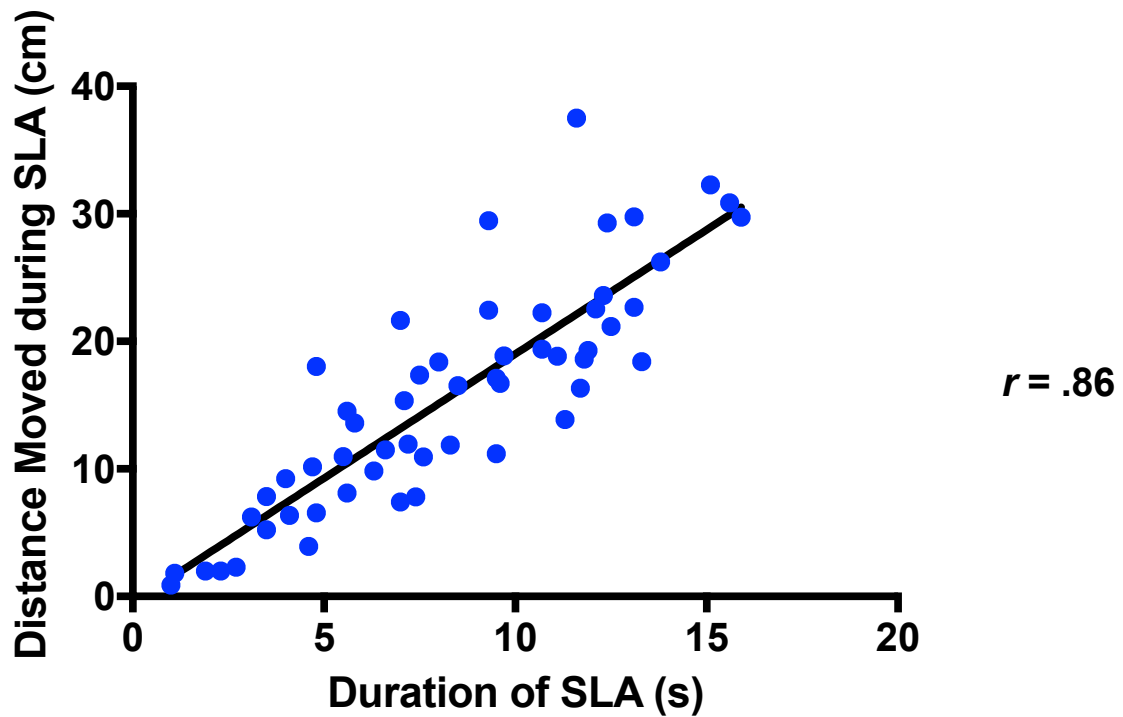


Figure 15. In EA + PTX flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .86$, $p < .001$.

There was a significant main effect for diet, $F(1, 202) = 6.51, p < .001$. The Bonferroni *post hoc* test revealed that velocity of SLA was significantly lower for the EA + PTX condition ($M = 1.83$ cm/s) when compared to the control ($M = 2.46$ cm/s) ($p < .05$) and EA ($M = 2.39$ cm/s) ($p < .05$) conditions (see Figure 16). Velocity of SLA was significantly lower for the EA + FL condition ($M = 1.82$ cm/s) when compared to the control ($M = 2.46$ cm/s) ($p < .05$) and EA ($M = 2.39$ cm/s) ($p < .05$) conditions (see Figure 16). The EA condition ($M = 2.39$ cm/s) did not significantly differ from the control condition ($M = 2.46$ cm/s) ($p > .05$) (see Figure 16).

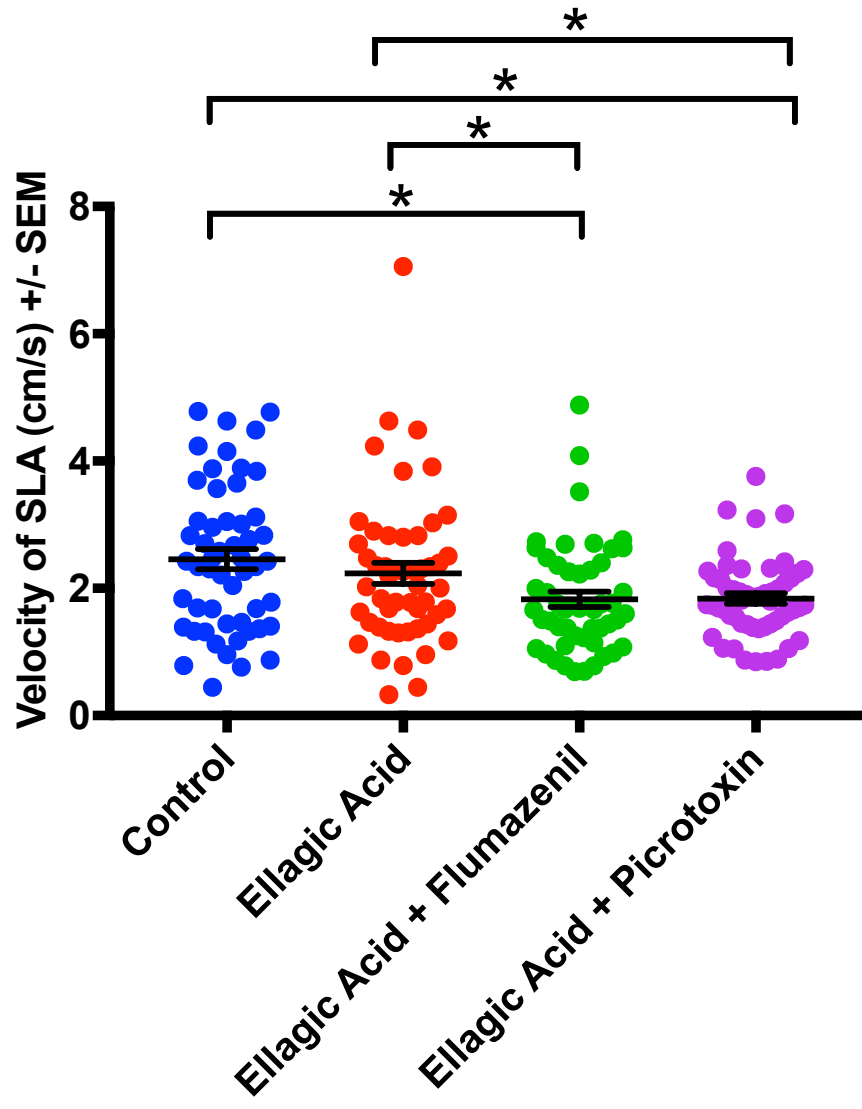


Figure 16. EA + PTX flies had significantly lower velocity of SLA when compared to the EA and control conditions. EA + FL flies had significantly lower velocity of SLA when compared to the EA and control conditions. $*p < .05$

Interaction Effect of Genotype and Diet

There was a significant interaction effect between genotype and diet on duration of SLA, $F(3, 202) = 4.44, p < .05$. The Bonferroni *post hoc* test revealed that duration of SLA in the EA condition was lower for *eas* flies ($M = 3.19$ s) when compared to *bss* flies ($M = 5.43$ s) ($p < .05$) (see Figure 17). Duration of SLA in the EA + PTX condition was higher for *eas* flies ($M = 9.18$ s) when compared to *bss* flies ($M = 7.30$ s) ($p < .05$) (see Figure 17).

There were no significant differences in duration of SLA among the control ($M = 5.65$ s), EA ($M = 5.43$ s), EA + FL ($M = 6.22$ s), and EA + PTX ($M = 7.30$ s) conditions for *bss* flies ($p > .05$) (see Figure 17). In *eas* flies, duration of SLA was higher for the EA + FL condition ($M = 6.69$ s) when compared to the control ($M = 4.07$ s) ($p < .05$) and EA ($M = 3.19$ s) ($p < .01$) conditions (see Figure 17). Duration of SLA was higher for the EA + PTX condition ($M = 9.18$ s) when compared to the control ($M = 4.07$ s) ($p < .001$), EA ($M = 3.19$ s) ($p < .001$), and EA + FL ($M = 6.69$ s) ($p < .05$) conditions for *eas* flies (see Figure 17). The EA condition ($M = 3.19$ s) did not differ significantly from the control condition ($M = 4.07$ s) in *eas* flies ($p > .05$) (see Figure 17).

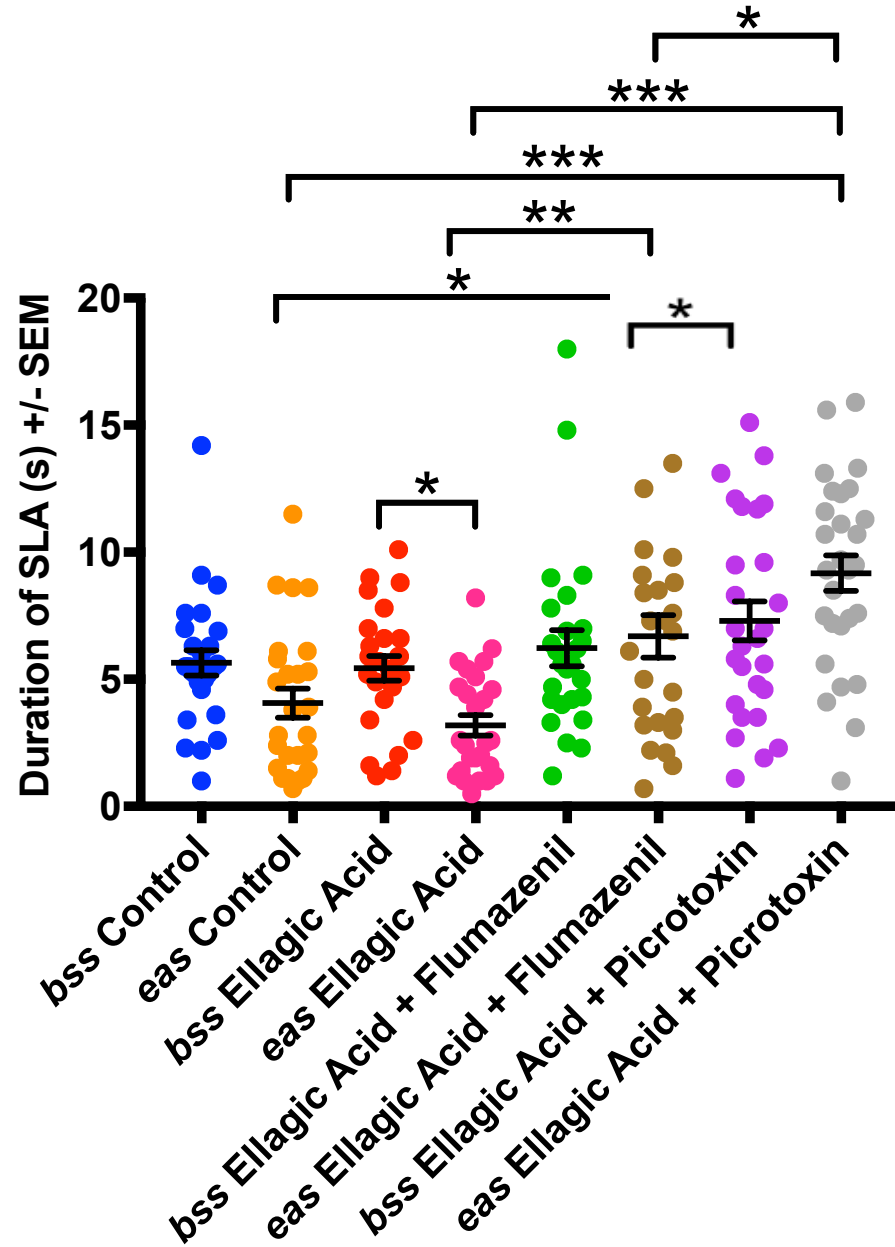


Figure 17. In the EA condition, *eas* flies exhibited significantly less duration of SLA when compared to *bss* flies. In the EA + PTX condition, *eas* flies' duration of SLA was significantly higher when compared to *bss* flies. Among *bss* flies, there were no significant differences in duration of SLA. In *eas* flies, duration of SLA was significantly higher for the EA + FL and EA + PTX diets when compared to the EA and control conditions. Duration of SLA was also higher for the EA + PTX diet when compared to the EA + FL condition. There was no significant difference between the EA and control conditions. * $p < .05$; ** $p < .01$; *** $p < .001$

There was a significant interaction effect between genotype and diet on distance moved during SLA, $F(3, 202) = 3.74, p < .05$. The Bonferroni *post hoc* test revealed that distance moved during SLA in the control condition was greater for *bss* flies ($M = 16.87$ cm) when compared to *eas* flies ($M = 9.27$) ($p < .05$) (see Figure 18).

There were no significant differences in distance moved during SLA among the control ($M = 16.87$ cm), EA ($M = 13.62$ cm), EA + FL ($M = 13.07$ cm), and EA + PTX ($M = 13.28$ cm) conditions for *bss* flies ($p > .05$) (see Figure 18). In *eas* flies, distance moved during SLA was greater for the EA + PTX condition ($M = 17.86$ cm) when compared to the control ($M = 9.27$ cm) ($p < .05$) and EA ($M = 8.91$ cm) ($p < .05$) conditions (see Figure 18). The EA condition ($M = 8.91$ cm) did not significantly differ from the control condition ($M = 9.27$ cm) in *eas* flies ($p > .05$) (see Figure 18).

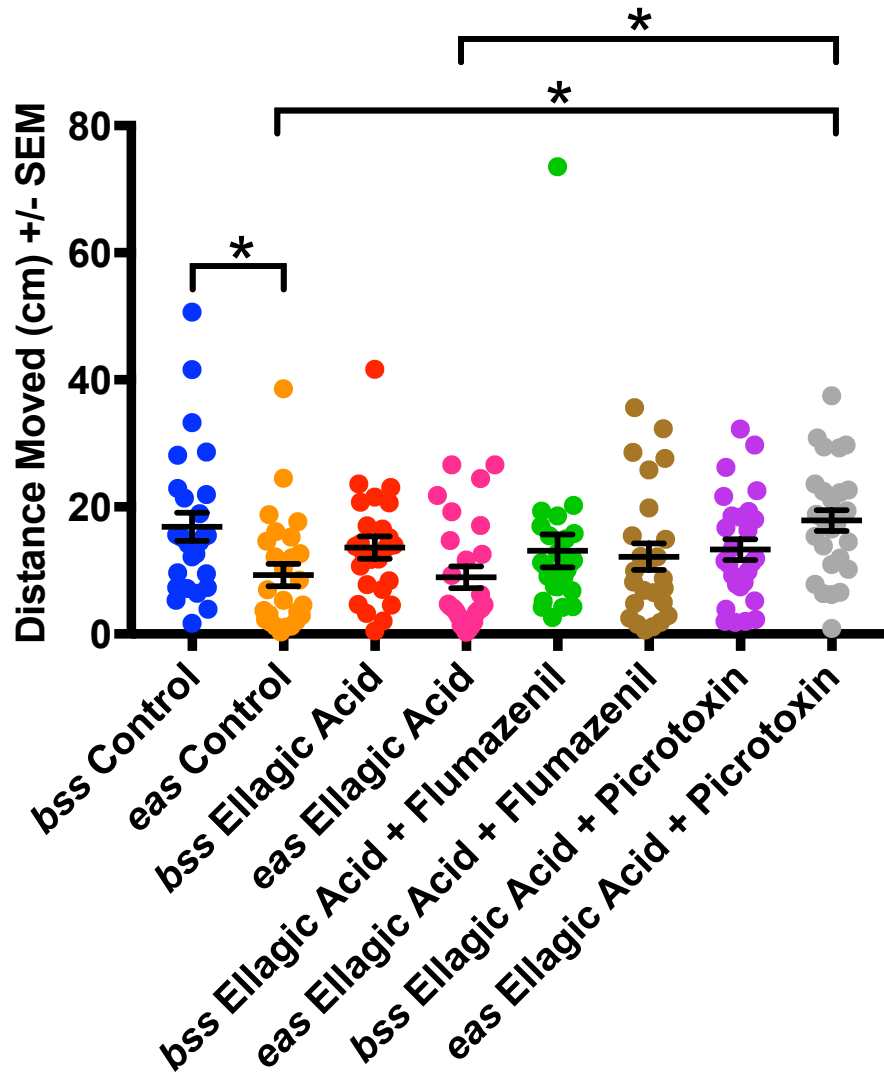


Figure 18. In the control condition, *bss* flies exhibited greater distance moved during SLA when compared *eas* flies. Among *bss* flies, there were no significant differences in distance moved during SLA. In *eas* flies, distance moved during SLA was significantly higher for the EA + PTX diet when compared to the EA and control diets. There was no significant difference between the EA and control conditions. $*p < .05$

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *bss* control flies, $r = .90$, $p < .001$ (see Figure 19).

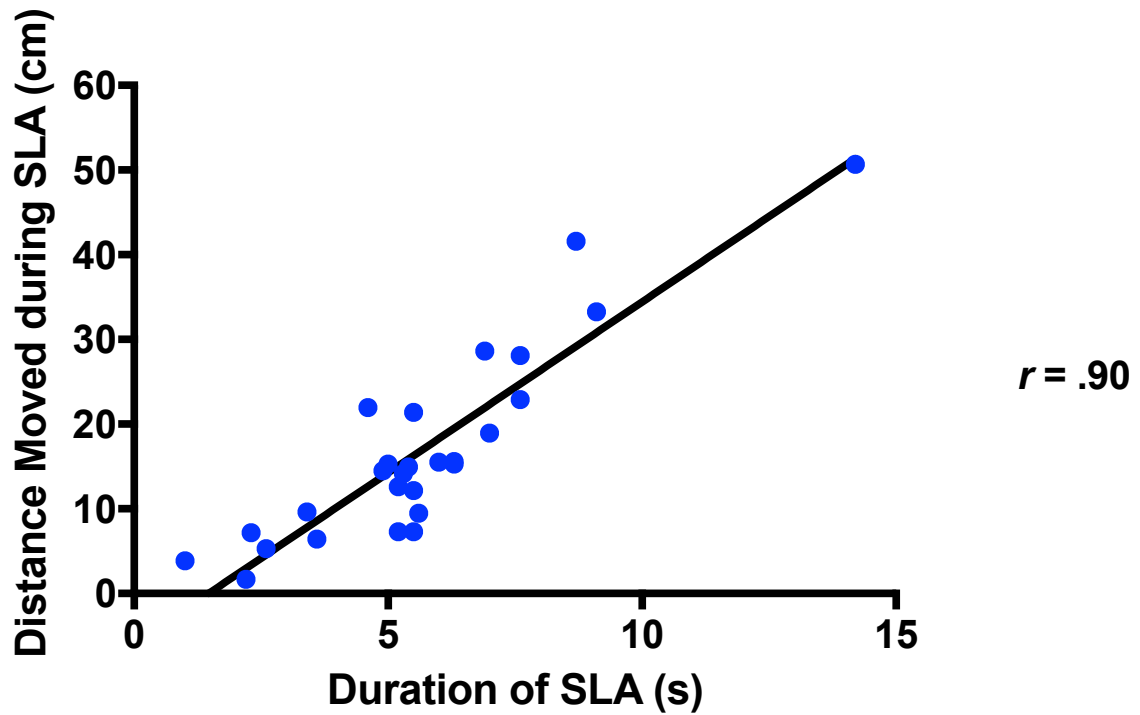


Figure 19. In *bss* control flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .90$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *eas* control flies, $r = .73$, $p < .001$ (see Figure 20).

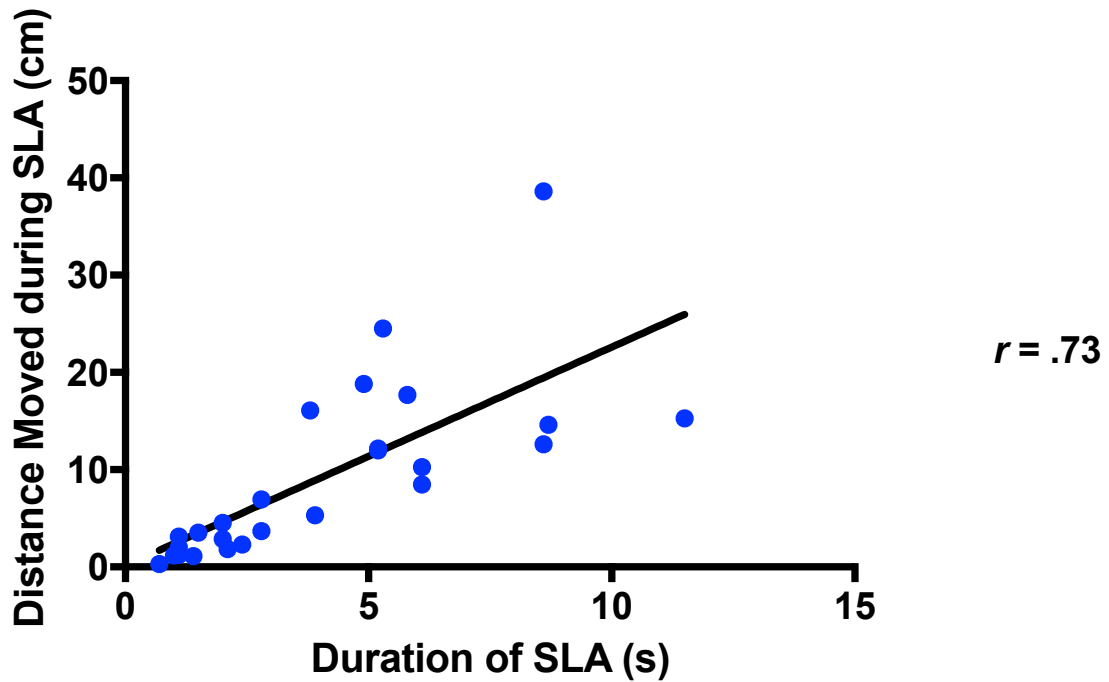


Figure 20. In *eas* control flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .73$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *bss* EA flies, $r = .68$, $p < .001$ (see Figure 21).

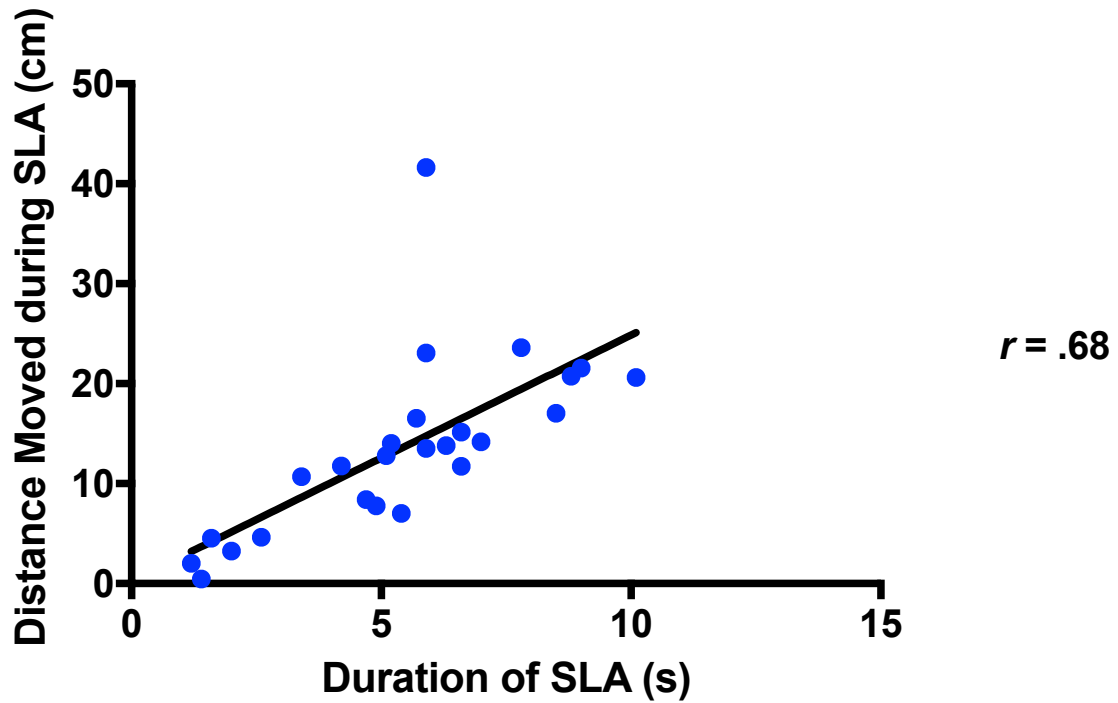


Figure 21. In *bss* EA flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .68$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *eas* EA flies, $r = .86$, $p < .001$ (see Figure 22).

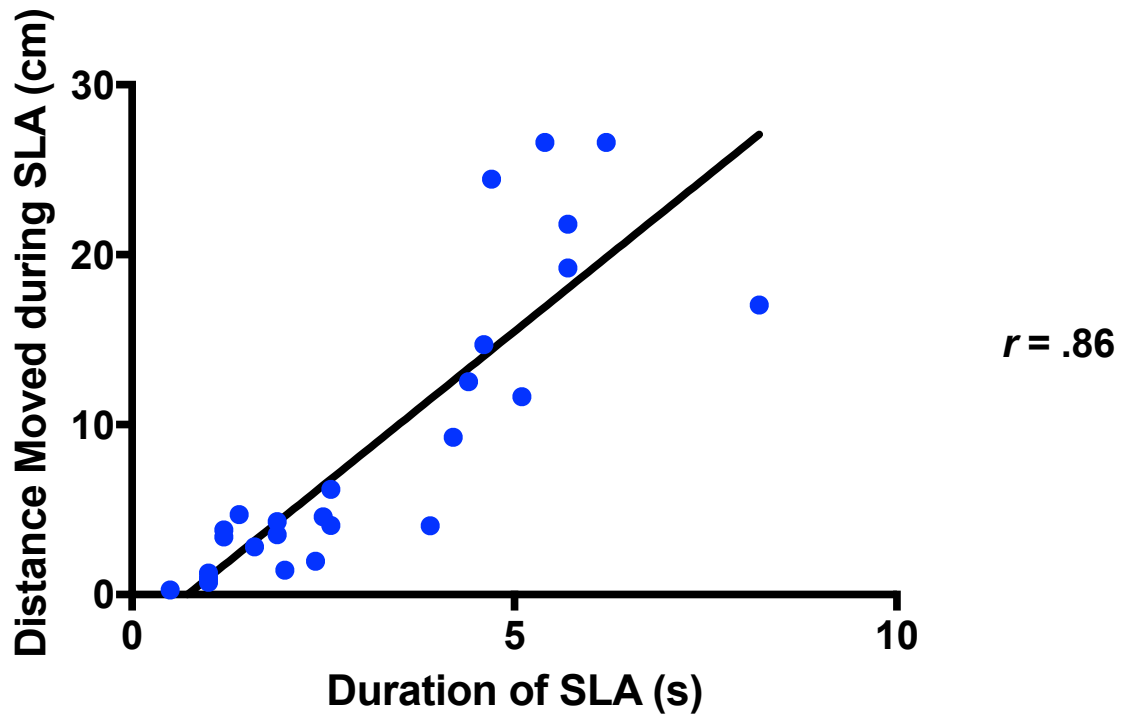


Figure 22. In *eas* EA flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .86$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *bss* EA + FL flies, $r = .81$, $p < .001$ (see Figure 23).

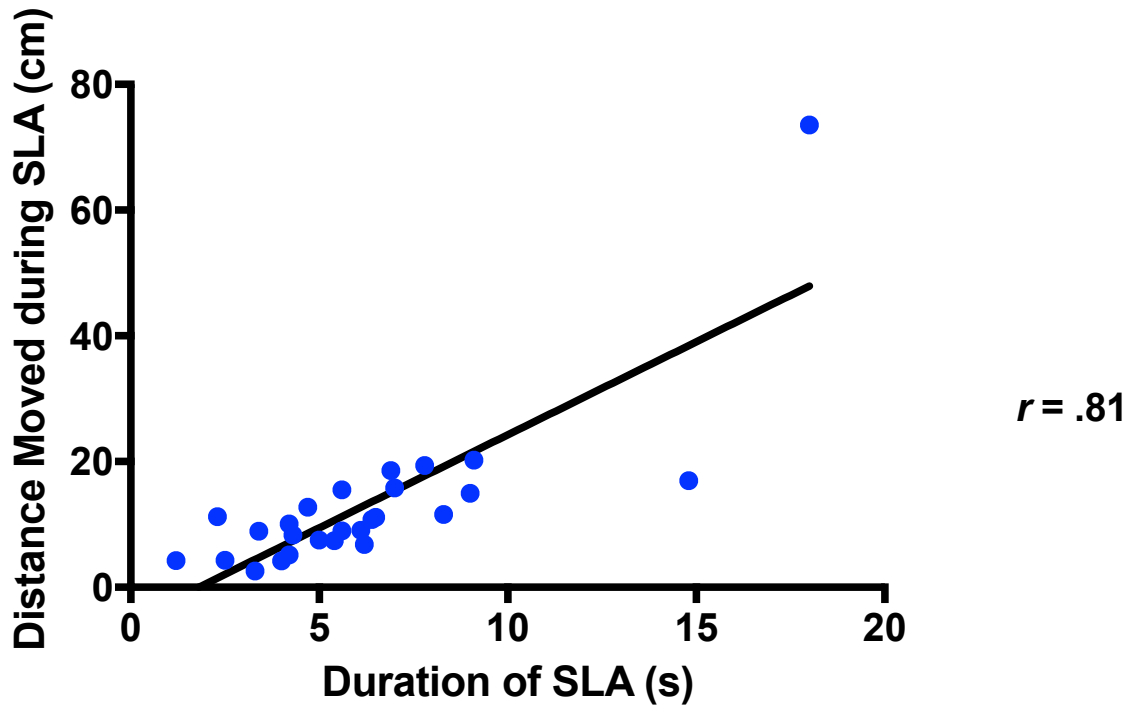


Figure 23. In *bss* EA + FL flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .81$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *eas* EA + FL flies, $r = .93$, $p < .001$ (see Figure 24).

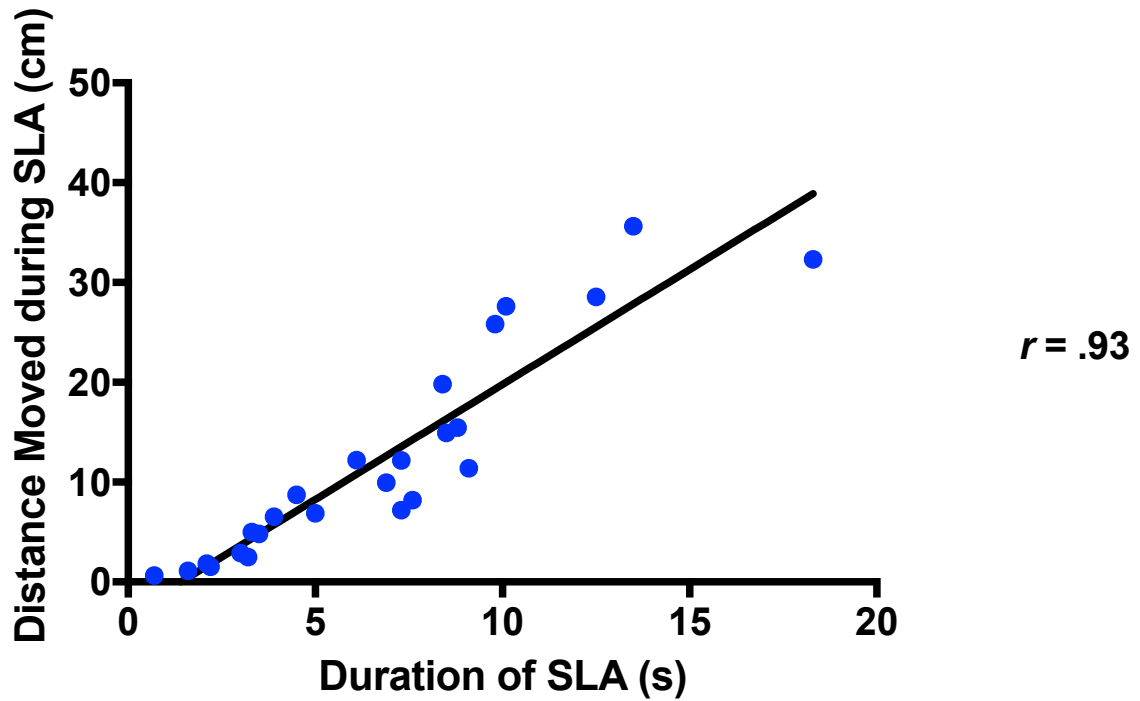


Figure 24. In *eas* EA + FL flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .93$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *bss* EA + PTX flies, $r = .88$, $p < .001$ (see Figure 25).

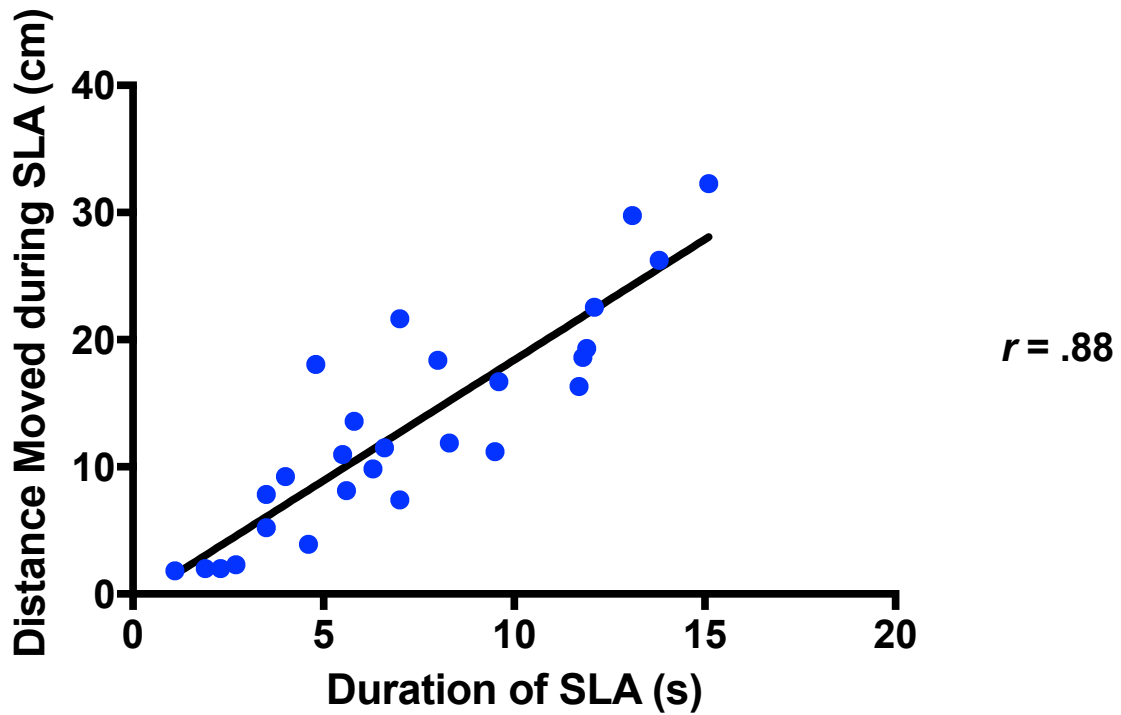


Figure 25. In *bss* EA + PTX flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .88$, $p < .001$.

There was a significant, positive correlation between distance moved during SLA and duration of SLA among *eas* EA + PTX flies, $r = .82$, $p < .001$ (see Figure 26).

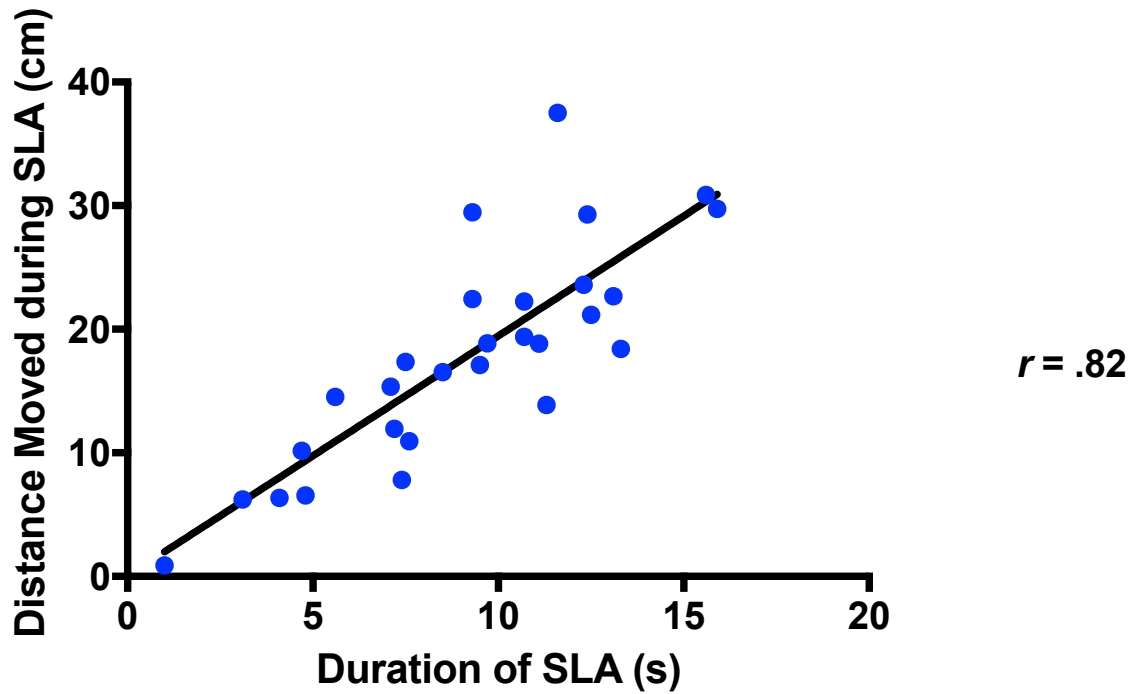


Figure 26. In *eas* EA + PTX flies, there was a significant, direct relationship between distance moved during SLA and duration of SLA, $r = .82$, $p < .001$.

There was a significant interaction effect between genotype and diet, $F(3, 202) = 4.44$, $p < .05$. The Bonferroni *post hoc* test revealed that velocity of SLA in the control condition was higher for *bss* flies ($M = 2.84$ cm/s) when compared to *eas* flies ($M = 2.06$ cm/s) ($p < .01$) (see Figure 27).

Velocity of SLA in the EA + FL condition ($M = 2.08$ cm/s) was significantly lower when compared to the control condition ($M = 2.84$ cm/s) ($p < .05$) for *bss* flies (see Figure 27). Velocity of SLA for the EA + PTX condition ($M = 1.76$ cm/s) was significantly lower when compared to the control condition ($M = 2.84$ cm/s) ($p < .001$) for *bss* flies (see Figure 27). The EA condition ($M = 2.41$ cm/s) did not significantly differ from the control condition ($M = 2.84$ cm/s) in *bss* flies ($p > .05$) (see Figure 27). In *eas* flies, the EA + FL condition ($M = 1.56$ cm/sec) velocity of SLA was significantly lower when compared to the EA condition ($M = 2.06$ cm/sec) ($p < .05$) (see Figure 27). The EA condition did not significantly differ from the control condition in *eas* flies ($p > .05$) (see Figure 27).

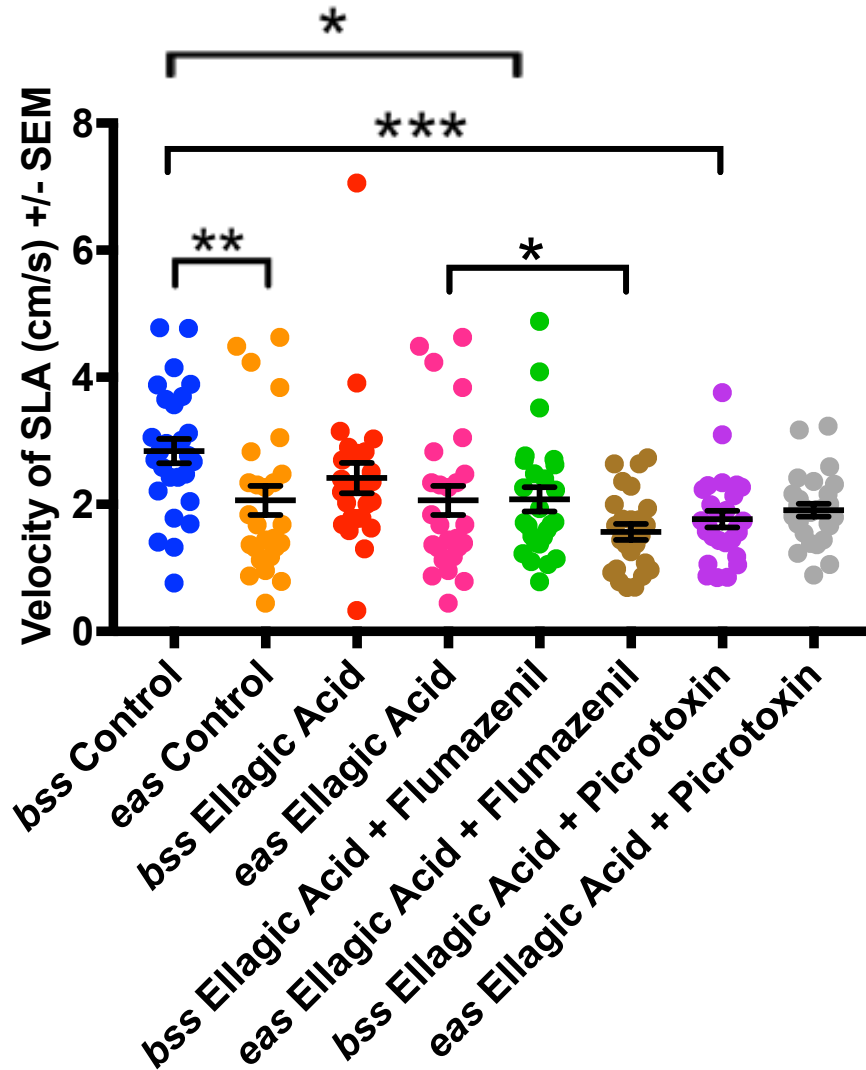


Figure 27. In the control condition, *bss* flies had significantly higher velocity of SLA when compared *eas* flies. Among *bss* flies, velocity of SLA was significantly lower for the EA + FL and EA + PTX diets when compared to the control condition. There was no significant difference between the EA and control conditions. In *eas* flies, velocity of SLA was significantly lower for the EA + FL diet when compared to the EA condition. There was no significant difference between the EA and control conditions. * $p < .05$; ** $p < .01$; *** $p < .001$

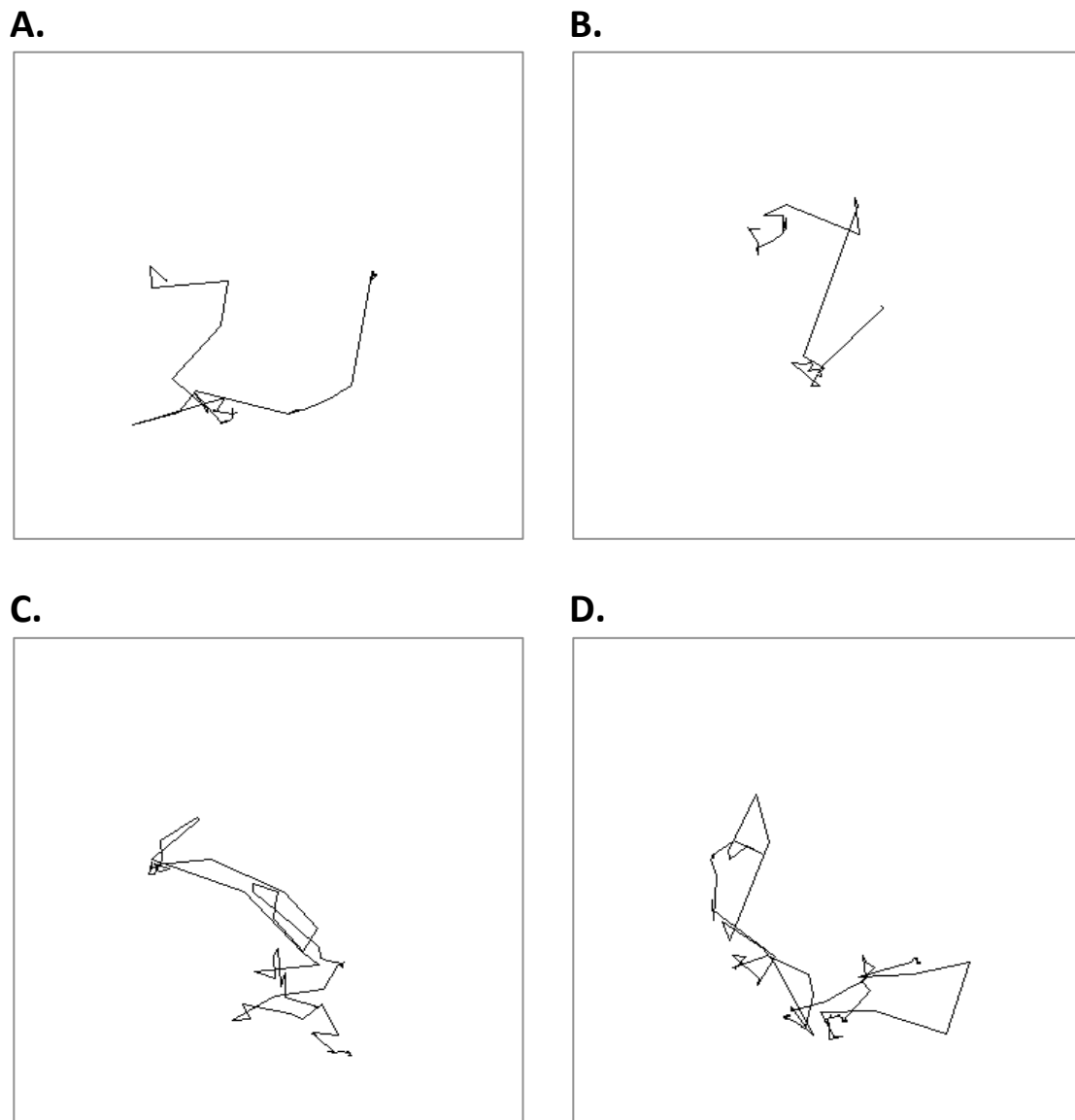


Figure 28. Representative path lengths of individual *eas* flies during SLA. (A) Path of an *eas* control mutant. (B) Path of an *eas* EA mutant. (C) Path of an *eas* EA + FL mutant. (D) Path of an *eas* EA + PTX mutant.

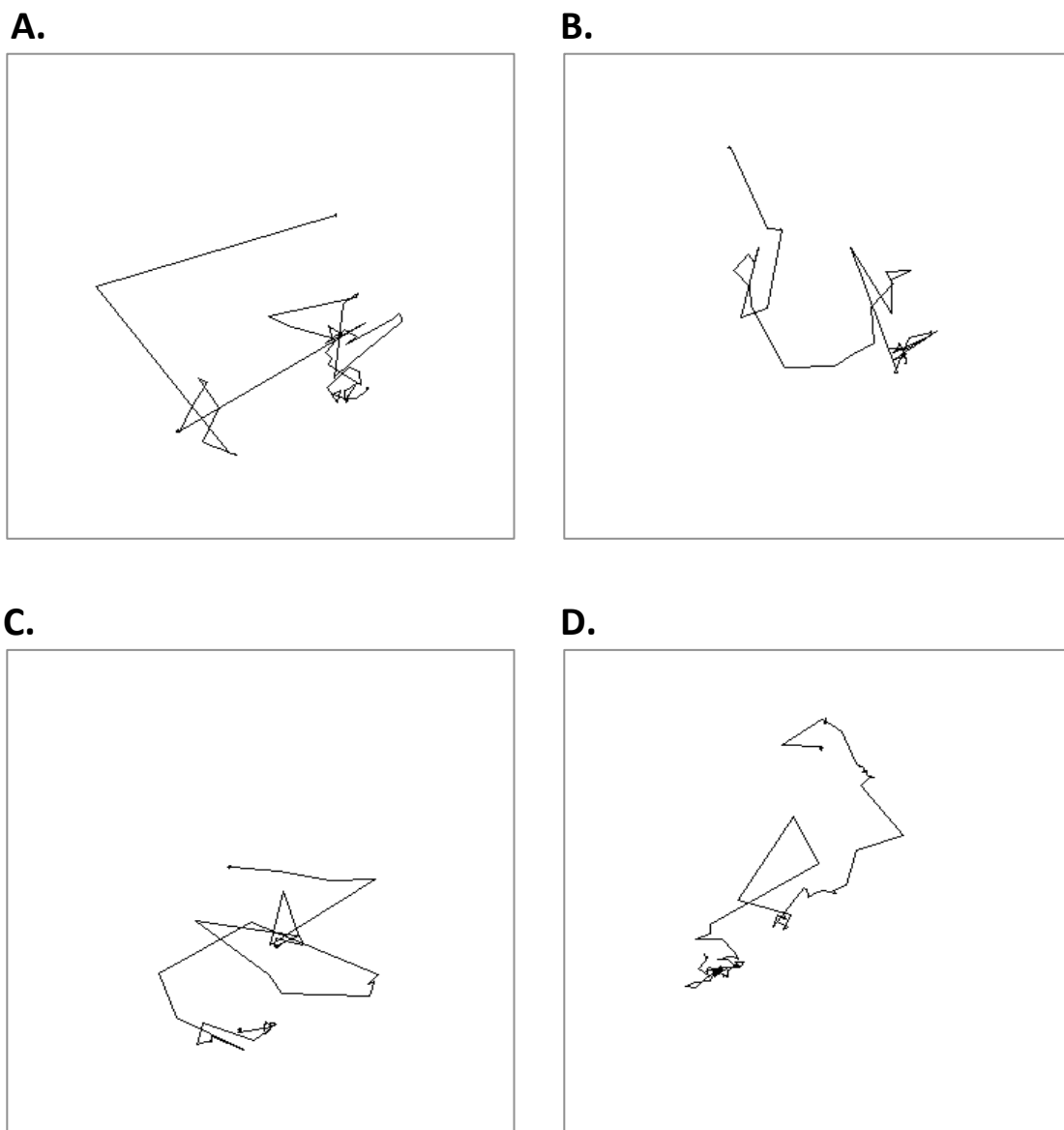


Figure 29. Representative path lengths of individual *bss* flies during SLA. (A) Path of a *bss* control mutant. (B) Path of a *bss* EA mutant. (C) Path of a *bss* EA + FL mutant. (D) Path of a *bss* EA + PTX mutant.

CHAPTER FOUR

DISCUSSION

The first aim of the study was to examine the effects of seizure modeling with mechanical shock in *bss* and *eas* flies. Vortexing for 10 seconds reliably induced seizures in 93.8% of both strains. Duration and distance moved were positively associated and effectively assessed SLA; however, using velocity as a dependent variable was a limitation of this model. Velocity was susceptible to being depressed as duration of SLA severity increased. As a result, this variable did not always truly reflect heightened SLA intensity due to its dependency on duration and distance moved expressed as a ratio (cm/s).

Although main effects demonstrated that *bss* and *eas* strains had similar SLA overall, examining both control groups showed that *bss* flies exhibited almost twice as much SLA as *eas* flies with respect to distance moved (~16 cm vs ~9 cm). This provides additional support for research indicating that *bss* flies exhibit severe tonic-clonic SLA (Parker, Padilla, et al., 2011). These heterogeneous behavioral phenotypes (see Figures 28A and 29A) may be reflective of the differences in mutations affecting voltage-gated sodium channels in *bss* flies and phospholipid membrane composition in *eas* flies.

The next aim was to explore the anticonvulsant effects of EA on SLA in both strains of mutants. In *bss* and *eas* flies, EA reduced SLA when compared to controls; however these effects were non-significant. When comparing both strains, *eas* flies exhibited significantly less duration of SLA than *bss* flies (~3 s vs ~5 s). This is consistent with prior research, which demonstrated that *bss* flies are generally more resistant to the effects of anticonvulsant drugs than *eas* flies (Parker, Padilla, et al., 2011;

Song & Tanouye, 2008). These findings support that SLA in *bss* flies models drug-resistant seizures observed in intractable epilepsy with tonic-clonic seizures and severe myoclonic epilepsy which in both instances result from the mutation of the human Nav SCN1A (Parker, Padilla, et al., 2011). In addition, this novel application of EA demonstrates that utilizing Bang-Sensitive mutants with varying levels of seizure-sensitivity provide an effective drug-screening tool for refractory epilepsy in humans. Moreover, compounds found in plants may provide prophylactic benefits with regard to seizure severity.

The final aim was to determine the potential mechanism of action for EA by blocking its anticonvulsant effects through GABA antagonists. In *bss* flies, the EA + FL and EA + PTX diets slightly increased duration of SLA when compared to control and EA conditions; however, this effect was non-significant. Among *eas* flies, both GABA antagonists significantly increased SLA when compared to control and EA diets, as PTX exhibited the strongest convulsant effect.

In examining genotype differences, PTX was also more effective for *eas* flies than *bss* flies with respect to inducing SLA following mechanical shock. PTX's strong convulsant effect in this study aligned with prior research demonstrating that this GABA antagonist significantly increased SLA in wild-type *Drosophila* larvae (Canton-S strain) through a GABA_A receptor pathway (Stilwell et al., 2006). With respect to FL, this research provides evidence that this GABA antagonist may be useful as a pro-convulsant in *Drosophila* seizure modeling, as no studies to date have employed this approach. Overall, these findings with two types of GABA antagonists suggest that EA's anticonvulsant effect involves GABAergic systems in a strain-specific manner and may

be more likely mediated through a GABA_A receptor site than benzodiazepine site.

Future Directions

For prospective studies, additional PTX and FL only conditions need to be administered at a range of doses and compared to control diets to further clarify the convulsant properties of these two GABA antagonists in both *bss* and *eas* mutants. Non-seizure-sensitive flies (i.e., Canton-S strain) should also be vortexed to confirm that they do not exhibit SLA after mechanical shock. Next, these flies should be administered pro-convulsants like PTX and FL to determine if this makes them vulnerable to seizures after vortexing. Then, EA could be administered to determine if this potential seizure susceptibility can be ameliorated.

Future research should also include examining the effect of EA at a higher range of doses to determine if this leads to greater reductions in SLA, especially for *bss* flies that exhibited a non-significant decrease in seizure behavior. Additionally, EA should be administered in combination with other pomegranate polyphenols to explore any synergistic effects, as Seeram et al. (2005) demonstrated that pomegranate juice can have greater bioactivity when compared to its purified polyphenols. Moreover, this analysis is warranted as research from the LLU Behavioral Neuroscience Lab has demonstrated the beneficial effects of pomegranate juice for memory in humans following heart surgery (Ropacki, Patel, & Hartman, 2013) and for Alzheimer's disease pathology (Hartman et al., 2006) and radiation (Dulcich & Hartman, 2013) in rodent models.

Lastly, because pharmaco-resistant seizures in TLE are associated with cognitive deficits as well as with hippocampal sclerosis and damage to other regions of the brain,

EA's neuro-protective properties (Dolatshahi et al., 2015; Farbood et al., 2015) should be explored with this model. Histological analyses along with learning and memory assays could prove beneficial for investigating the relationships among SLA, brain cells, and cognitive functioning following the administration of EA, FL, and PTX.

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